WATER-SOLUBLE CARBOHYDRATES OF GRASS SEEDS

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GENERAL INTRODUCTION

There is abundant reason for widespread interest in grasses. The use of wheat in the preparation of flour, of barley in the brewing industry and of hay as an animal feeding-stuff are perhaps only some of the most obvious examples of the economic importance of the Gramineae. To the biochemist, considerable importance is attached to investigation of the processes of growth involved in members of this very distinct botanical family.

It is not surprising that much of this interest should be focussed, in view of their high content and economic importance, on the carbohydrates. Such investigation, both on the vegetative parts of the plant and also on the seeds, has been carried out from a chemical and from a biochemical point of view. Chemical analysis has sought to characterise the molecular entities to be found in the plants, and although it is a matter of great difficulty to effect fractionation of different molecular species, the basic, if not detailed, distribution of those has been reasonably well established. The biochemist seeks to establish what function such carbohydrates do perform in the plant during its growth, bearing in mind that the presence of any chemical substance may not invariably prove that it is necessary for the life of the plant. The extensive investigations on those plants containing alkaloids provide an obvious example

of this circumstance, for, although future work may establish some evidence to the contrary, it has not been possible to distinguish any function performed by those substances in the plant.

Although the work to be described is concerned with the seeds of grasses, the close relationship between the seeds on the one hand and, on the other hand, the stems and leaves of the plant should not be ignored. In the growing plant, the leaf is the centre of photosynthetic action and sucrose is the main product of synthesis. This carbohydrate is transported through the plant and made available for respiration and for formation of various plant tissues. It appears that some of this carbohydrate is stored, temporarily, in the form of fructosan molecules. Furthermore, in addition to the more labile carbohydrates in the stems, cellulose and hemicellulosic materials are constituents which presumably lend rigidity to the plant. During the period of growth vast changes occur in the absolute and relative quantities of those carbohydrates, and investigation of such changes has attracted considerable biochemical attention.

A very different content of carbohydrates is present in the seeds of the grasses, for it is from the seed that growth will begin and for this to occur, sufficient substrate must be available. For obvious

reasons of industrial importance, it is the grains of the common cereals which have attracted attention, and, of these, barley is perhaps the most widely investigated. In the cereals, the bulk of the reserve material is carbohydrate in nature in contrast with some seeds, for example, almond or cotton seeds, in which fatty material is almost exclusively utilised. Accordingly, although the structural carbohydrates, namely cellulose and hemicellulose are detected, there is invariably present a large quantity of starch, which is situated in the endosperm of the seed and is the major food reserve. The more readily available substrates include sugars, primarily sucrose, and a small quantity of fatty materials. As to the starches of the common cereals, chemical investigation shows them to consist of mixtures of amylose and amylopectin. Very close similarity is found amongst those, with in the main rather small differences in relative amounts of amylose and amylopectin being the most distinct point of contrast.

Purpose of the Work

In view of the restricted information available, concerning the carbohydrate content of the seeds of wild species of the grass family, it is the intention in this investigation to consider some, at least, of the carbohydrates to be found in them. So

that a relatively wide survey of the Gramineae could be carried out, investigation was restricted to the water-soluble carbohydrates present, starch for the purpose of this work being excluded from this definition. In effect, therefore, two groups of molecular species were under investigation, the first of those being the sugars, and the other being the high molecular material extracted by water and expected to resemble the cereal gums (Preece & Hobkirk, 1953).

The purpose of the work is, broadly speaking, three-fold; first to analyse those materials obtained from grass-seeds, with the intention of establishing their nature, and second to derive biochemical information from inspection of the results obtained. Finally, the possibility of obtaining information helpful to the taxonomy of the Gramineae is to be considered.

MATERIALS

Grass seeds were obtained from a wide variety of sources; approximately 40% of those were commercial samples. The remaining samples, which were obtained principally from the vicinity of Edinburgh, but in some cases from other locations, were dried in the laboratory before use. Only one sample, *Setaria italica* (millet), was not a British grass. Table 1 consists of a list of those grasses investigated giving details of where they were obtained and their 1000-corn weight.

Table I

Materials used.

Tribe*	Genus and Species*	Dry weight of 1000 seeds (as analysed)	Origin of Sample
Bromeae	Bromus sterilis L.	8.29 g.(20% husk)	Waste ground, Edinburgh
	Bromus mollis L.	3.76 g.	Waste ground, Edinburgh
	Bromus asper Murr	4.85 g.	Woodland, Bridge of Allen
Brachypodieae	Brachypodium sylvaticum (Huds) Beauv.	4.12 g.(25% husk)	Woodland, Bridge of Allen
Hordeae	Agropyron repens (L.) Beauv.	3.59 g.(30% husk)	Waste ground, Edinburgh
	Elymus arenarius L.	20.4 g.(26% husk)	Sand dunes, Longniddry
Glycerieae	Glyceria plicata Fries	0.86 g. -	Pond, West Edinburgh
Festuceae	Festuca pratensis Huds.	2.24 g. -	Commercial
	Lolium perenne L.	1.99 g. (19% husk)	Commercial
	Poa trivialis L.	0.15 g. -	Commercial
	Dactylis glomerata L.	0.73 g. -	Commercial
	Cynosurus cristatus L.	0.54 g. -	Commercial
Aveneae	Arrhenatherum elatius (L.) J & C. Presl	2.56 g. (30% husk)	Waste ground, Edinburgh
	Avena fatua L.	22.4 g. (48% husk)	Arable land, Rothamsted
	Holcus lanatus L.	0.34 g. (shelled)	Commercial
	Anthoxanthum odoratum L.	0.53 g. (40% husk)	Wood and heath, Bridge of Allen
Phalarideae	Phalaris canariensis L.	6.80 g. -	Commercial
Agrostideae	Ammophila arenaria L.	1.70 g. -	Sand dunes, Gullane
	Agrostis canina L.	0.06 g. -	Commercial
	Phleum pratense L.	0.41 g. -	Commercial
Nardeae	Nardus stricta L.	0.68 g. -	Various moors East Scotland
Danthonieae	Molinia caerulea (L.) Moench	0.35 g. -	Damp moorland East and West Scotland
	Sieglingia decumbens/		

Table I (contd.)

Tribe*	Genus and Species*	Dry weight of 1000 seeds (as analysed)	Origin of Sample
	Sieglingia decumbens (L.) Bernh.	2.34 g. -	Heath, Fort William
Spartineae	Spartina townsendii	10.02 g.(41% husk)	Southampton Water
Paniceae	Setaria italica (L.) Beauv.	4.63 g.	Commercial

*Classification as in Hubbard (1954).

The following grasses throughout the course of this work may be referred to by their common names.

Grass	Common Name
Festuca pratensis	Tall fescue
Lolium perenne	Perennial Rye-grass
Poa trivialis	Meadow grass
Dactylis glomerata	Cocksfoot
Cynosurus cristatus	Crested Dog's-tail
Avena fatua	Wild oats
Phalaris canariensis	Canary seed
Phleum pratense	Timothy-grass
Setaria italica	Millet seed

SECTION I

SUGARS AND OLIGOSACCHARIDES

INTRODUCTION

Throughout the plant kingdom, sugars and oligosaccharides are of universal occurrence and can be detected in all parts of the plant, in widely differing amounts. The high content - about 17% - of sucrose present in sugar cane becomes of the highest economic importance although it is more usual to detect amounts of sugar of the order of 1 or 2%, the excess sugar normally being converted to some storage polysaccharide. However, stachyose is obtained from the rhizomes of Stachys tubifera in yields amounting to 5% while sugar beet yields up to 16% of sucrose and as little as 0.5% of raffinose.

Little detailed information is available concerning the sugar content of the stems and leaves of plants, though reference is frequently made during investigation to the detection, if not analysis, of sugars; and in any event, wide fluctuations occur with varying environmental conditions (see e.g. Yemm, 1935). Perhaps more qualitative information is available with regard to those sugars and oligosaccharides found in the seeds of plants; logically so, in view of the undoubted importance of sugars during the early stage of germination. It is clear that sucrose is invariably present and there seems little doubt that this sugar is most vitally concerned in the metabolism of germination. As to monosaccharide content,

fructose and glucose are generally detected, but relatively in small quantity, while other monosaccharides are effectively absent - small exception to this statement might be made on account of reports of galactose and arabinose in trace quantities.

It is of interest to consider those oligosaccharides, other than sucrose, which may occur in seeds. The almost universal absence of maltose and cellobiose and of any degradation products of starch and cellulose is striking, and similarly pentose oligosaccharides are only infrequently encountered. A comparatively restricted "family" comprises those oligosaccharides most likely to be found in plants; this "Raffinose family" of oligosaccharides has recently been reviewed very fully, (French, 1954). The trisaccharide raffinose may be represented as α galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)β-D-fructofuranoside, consisting, therefore, of one galactose residue linked to a sucrose structure. Stachyose, verbascose, and possibly ajugose comprise the succeeding members of a short homologous series which is built on raffinose by the addition of galactose residues, joined by α(1→6) linkages to the terminal galactose residue of raffinose. The trisaccharide planteose, occasionally detected in plants, bears some

similarity to raffinose. It differs from raffinose in that its galactose residue is linked to the fructose rather than the glucose residue. Of the restricted number of other oligosaccharides reported, the most common are the trisaccharides gentianose and melezitose and the disaccharide, trehalose. Manninotriose and melibiose, degradation products of the raffinose type oligosaccharide, are also found free in a very restricted number of plants.

Although O'Sullivan (1886) was one of the first to investigate the sugar content of the seeds of barley, very little detailed information was available until recently concerning the sugars of other cereal grains. O'Sullivan, however, was able to establish that sucrose was present in the barley grain; moreover he succeeded in identifying raffinose. In recent years, MacLeod & Preece (1954) using methods of paper chromatography have carried out a comparative investigation of those sugars and oligosaccharides, detected in the seeds of five common cereals - barley, wheat, rye, oats, and maize. Those sugars and oligosaccharides are the carbohydrates extracted in boiling 80% ethanol and appear to include oligosaccharides containing up to 10 sugar units. In a restricted survey of this nature, generalisations must be made with reserve, but, nevertheless, striking points of similarity and of

contrast were apparent. Small quantities of monosaccharides were invariably detected, in addition to sucrose and raffinose, while the most striking feature of comparison was the occurrence of a low-molecular fructosan series in wheat, rye and barley to the extent of 1-4%, the detection of not more than a trace of this series in oats and its complete absence from maize. It is, further, of interest to observe that the three fructosan-containing cereals are taxonomically homogeneous, all being members of the tribe Hordeae. The quantities of sucrose detected were of the order of 1% of the seed weight, raffinose 0.2-0.4% and reducing sugars of the order of 0.2%. Maltose, in trace, was also reported, but only in barley does it seem to exist to any extent at all.

MacLeod (1953), on further investigation of those low-molecular fructosans of barley, has successfully resolved the series into a number of oligosaccharides by use of charcoal column and paper chromatography. Each of those fructosans has been shown to contain a number of fructose residues in combination with one glucose residue. It is suggested that the glucose residue is in a terminal position and that in combination with the adjacent fructose moiety, it constitutes a sucrose structure. It is believed that increasing numbers of fructose

residues are linked to this basic sucrose structure, resulting in an homologous series of fructosans, though the nature of linkage cannot certainly be established without the employment of structural analysis. Structural analysis of kestose (Albon et al., 1953), a trisaccharide containing one glucose and two fructose residues has shown it to be O-α-D-Glucopyranosyl-(1→2)-O-β-D-Fructofuranosyl-(6→2)-β-D-Fructofuranoside but it is not certain that this sugar is identical with the trisaccharide member of the fructosan series. Irrespective of the fine structure of those fructosans, it appears that they constitute a somewhat distinct molecular group in comparison to fructosans, frequently reported, of the inulin and levan type. Those molecules may contain from ten to thirty residues, while the series under consideration is low-molecular, the first member of which is, in fact, sucrose, a disaccharide.

Added to this general analytical data, information is available concerning the metabolism of sugars and oligosaccharides during germination, and to a lesser extent during ripening. An examination of sugar content of barley grain during the latter stages of ripening (Harris & MacWilliam, 1954a) shows that some apparent utilization of sugars at this stage does occur. Monosaccharides, sucrose and

raffinose, increase to some extent over the last four weeks of ripening, while fructosan content decreases. It has been observed elsewhere that fructosans are absent from the ripening ears of maize but are present in oats, barley, rye and wheat. During the course of ripening, the fructosans of oats disappear or fall to a very low concentration, but these oligosaccharides persist in the remaining three cereals (Colin & Belval, 1923).

James, without the help and advantages of paper chromatography, carried out in 1940, a remarkable survey of sugar changes during the germination of barley and the early stages of seedling growth. His results refer to concentrations of monosaccharides as hexose, sucrose and raffinose at daily intervals during the growth of barley seedlings, both under normal conditions of growth and also on germination of previously excised embryos. In each case, a rapid utilisation of both sucrose and raffinose occurred until after two days, the concentration of those sugars in isolated embryos had fallen virtually to zero. When the entire grain was allowed to germinate, significant difference resulted in the sugar content of the embryo. Again, raffinose concentration fell to zero in one day, while sucrose content similarly was reduced to a small amount. Thereafter, however, a very considerable increase in

sucrose, but not in raffinose content occurred until there was, in fact, an overall increase in the quantity of sucrose. The implication of those observations appears to be that both sucrose and raffinose are utilised as substrates for germination at a very early stage, as shown by the sharp drop in their concentrations. The fact that sucrose content thereafter increases is still consistent with its continued utilisation, but clearly requires a considerable synthesis of the sugar from some reserve source; it is the starch content of the endosperm which provides this reserve. The precise mechanism involved in the transformation of starch to sucrose still remains obscure, although some understanding is being obtained of the mechanism of related processes, for example of the synthesis of starch from sucrose in the leaves of plants (Porter & May, 1954).

The complete disappearance of raffinose during germination has prompted MacLeod (1957) to reinvestigate this problem in the hope that analytical methods now available would allow a clearer understanding of the fate of this sugar. The findings of this work corroborate the earlier results of James, but draw attention to the interesting fact that raffinose does not appear to be utilised by seeds which have first been steeped anaerobically, though sucrose utilisation is even more rapid than in normal

aerobic germination. As to the fate of the sugar, there is no clear evidence of any direct synthesis from raffinose to a polysaccharide molecule, nor has it been possible to detect with certainty any products of degradation; the formation of a trace of free galactose is suspected, and this suggests the formation of sucrose and galactose, both of which presumably would be immediately available for use.

Further studies on the metabolism of sugars during the germination of barley during commercial brewery malting have been reported. In contrast with the laboratory experiments already referred to, a malting is successful at a temperature of about 15°C., several degrees lower than that used for most laboratory germinations, and growth is of much superior regularity. A qualitative and quantitative analysis (MacLeod, Travis & Wreay, 1953) carried out at daily intervals throughout the entire course of malting, provided substantiation of the laboratory investigation of James, and furthermore, because of the use of the paper chromatographic technique, allowed fractionation and investigation of the oligosaccharides present. The carbohydrates considered were those extracted by refluxing in 80% ethanol solution. Again sucrose content rose after a marked drop in the early stages while there was rapid and complete disappearance of raffinose. There was. in addition.

considerable increase in the concentration of monosaccharides, glucose increasing more than fructose, and the appearance of oligosaccharides of the maltose and maltotriose type, presumably as a result of the breakdown of starch, was observed. The fate of the fructosan series is difficult to assess because of the production of the "maltose" oligosaccharides, but an early drop in their concentration suggests their utilisation. It was possible to detect a subsequent rise in glucodifructose content at a later stage, suggesting the possibility of a further synthesis of fructosan series in parallel with the increase of sucrose. Further investigation, (Harris & MacWilliam, 1954b) similar in nature and using similar methods has substantiated those results in all but some quantitative detail. Those differences are attributable to minor differences in different grain samples, malted under different conditions.

The changes in concentrations of the simpler sugars, during malting, are therefore established with some certainty, but further more detailed analysis would be essential to provide similar data for the fructosan and "maltose type" oligosaccharides. The metabolic significance of those results is merely hinted at or imperfectly explained, but several salient points have emerged. It can be said with

certainty that sucrose is of cardinal importance during germination and it is generally agreed that it is this sugar which is most urgently required both for the synthesis of polysaccharides and also as a source of energy for respiration. Discussion of the mechanisms of synthesis is provided in a paper by Stacey (1956) in which the significance of a number of in vitro synthesis is considered. Reference is made to the utilisation of sucrose in such reactions as the bacterial synthesis of dextrans and further in the synthesis of an amylopectin-type of molecule. Though the synthesis of polysaccharides in nature does not necessarily follow the pattern of all successful laboratory syntheses, yet such experiments do demonstrate the probability of the occurrence of similar reactions. Stacey, moreover, points out that synthesis of any one polysaccharide molecule may be effected from more than one simple substrate, emphasising the possible utilisation of sugars other than sucrose.

That sugars, particularly sucrose, are involved in the processes of germination and that they are capable of acting as substrates for synthesis, has been certainly established. But, attempts to establish the nature and quantity of sugars required respectively for synthesis and respiration have met only with a restricted measure of success. James &

James (1940), investigating the problem of respiratory substrates in barley seedlings, concluded that the initial stages of respiration depended on slow oxidation of fatty material, but that at an early stage sucrose and other sugars become the major respiratory substrate. A slight drop in respiration (as measured by carbon dioxide production) was detected at the stage when embryonic sugar was exhausted, but this was soon overcome by endospermic material becoming available as a respiratory substrate. As to the relative quantities of carbohydrate material utilised for synthesis and respiratory purposes, Barnell (1937) measured transfer of carbohydrate from endosperm to seedling during a period of approximately 7 days. By that time, four-fifths of the endospermic carbohydrate had been utilised. It was observed that two-thirds of the carbohydrates utilised were located in the seedling while one-third had been lost through respiration.

From an historical view-point, those studies now discussed fall into three broad groups. The latter years of the 19th century saw the pioneer work which resulted in very considerable progress on qualitative analysis of sugar content, this period being followed by a time of relative inactivity, resulting largely from the lack of suitable and more exact methods of analysis. Since 1930, or thereabouts, interest has

revived not only in sugar content of seeds but in the metabolic functions of the sugars and in their distribution in the seed. Modern analytical methods overcome many, if not all, of those difficulties which curtailed early work and may allow a more comprehensive attack to be made on some of the remaining problems.

EXPERIMENTAL

The work of MacLeod & Preece (1954) has shown that distinct qualitative as well as quantitative differences exist in the sugar content of the seeds of a number of the common cereals. A similar investigation is now carried out on a much wider selection of the Gramineae. The analytical procedure has as far as possible been kept standard, so that the results obtained would lend themselves to comparison.

Preparation of Extracts

The methods of extraction and of analysis were essentially those described by MacLeod (1951). A minimum of 50 g. of seed were ground fairly finely in a coffee mill and added to at least three times its weight of boiling 80% ethanol. Many seed samples

absorbed so much alcohol that as much as five times their weight of alcohol was required. The seed was extracted under reflux for thirty minutes and after allowing the mixture to settle for a moment, the alcohol was decanted and filtered through a Buchner funnel. Any solid was returned to the flask and the seed again extracted with alcohol. In all four extractions were carried out. After the final extraction, the entire mixture was decanted into a Buchner funnel and allowed to drain. The alcoholic extract (a volume of 500-600 ml. would result from extraction of 50 g. seed) was left at least overnight at a low temperature to allow foreign material, much of it fatty in nature, to precipitate. After the removal of this material, the bulk of the alcohol was distilled off, during distillation the solution being shaken several times to help to coagulate any material which was settling out. The resultant aqueous extract was again left overnight and any solid removed. Concentration to a suitable volume (less than 100 ml. for a 50 g. extract) was carried out on a water bath and one further day allowed for clarification of the extract. A clear solution, frequently with a port-wine colour, was usually obtained.

Several extracts, notably that of wild oats, were particularly troublesome to obtain clear. Preliminary Soxhlet extraction was not employed because

of its restricted advantage and time consumption, while shaking the extract with, for example, petroleum ether or alumina cream was found to be of little advantage. In general, clear extracts could be obtained, if sufficient time was allowed for foreign material to coagulate. To safeguard against bacterial action, such delay was only permitted while alcohol was still present in the solution.

Total Reducing Sugar Content. After adjusting the volume of the solution to a known volume (e.g. 100 ml.), the total reducing sugar content was determined by use of the Somogyi micro-copper technique (Somogyi, 1945). Aliquots of the solutions, usually varying from one to five ml. were used so that determinations could be obtained within the optimum range for the method. In practice it was found that glucose and fructose were the only reducing sugars present with the exception of traces of galactose, arabinose and a reducing disaccharide, whose contribution to the reducing power of the extract was so small that it could safely be ignored. The total amount of glucose and fructose contained in a quantity of seeds could therefore be obtained. Thereafter estimation of the relative amounts of those monosaccharides and oligosaccharides (the method is described later) allowed calculation of the actual amount of each sugar present.

Separation and determination of sugars and oligosaccharides

The aqueous extract was concentrated to a syrup on a water-bath and the sugars were taken up in a small volume (about 10 ml.) of 50% ethanol, by prolonged stirring and shaking. The solution which almost invariably was coloured brown, was used for the preparation of chromatograms.

Qualitative investigation. Whatman No. 1 paper 57 cm. in length and 11.5 cm. wide was usually used for chromatograms. Sugar solutions were applied 10 cm. from one end of the paper which was then normally irrigated with butanol/acetic acid/water solvent using the descending chromatographic technique (Partridge, 1948). The chromatograms were suspended inside drain-pipes standing on and covered by glass plates, sealed with vaseline. Separation was carried out in three to four days in a controlled temperature of 19°C. Chromatograms were prepared by applying the sugar solution at regular intervals across the paper with a spot of control solution at each side of the paper. Those spots were dried at about 40°C. Raffinose, maltose, sucrose, glucose and fructose were used as reference sugars. After irrigation of the chromatograms, they were dried at 100°C. and then cut into strips. The control sugars were located by spraying with aniline oxalate and

heating at 100°C. for about five minutes. Paper strips containing the sugar mixture under investigation were sprayed with aniline oxalate to detect all sugars, with ∝ naphthol/phosphoric acid (Albon & Gross, 1950) to detect ketose sugars and a third strip was treated with silver nitrate in acetone and sprayed with alcoholic sodium hydroxide (Trevelyan et al., 1950) to detect reducing sugars. Aniline oxalate proved both reliable and extremely sensitive, while with care, the method of detection of ketoses was also reliable; the value of the spray for detecting reducing sugars was, however, found to be somewhat restricted, particularly for the detection of very small quantities, mainly because of a tendency for some reaction with non-reducing sugars.

The information gained by the use of those reagents along with comparison of chromatographic mobility of sugars to those of reference sugars, does not serve as a conclusive identification of any sugar, but it does provide valuable indications as to their nature. In the case of, for example, glucose, fructose and sucrose, sugars which are reasonably certain to be detected, this information was regarded as being sufficiently diagnostic. It was necessary, however, to extend investigations on the more complex oligosaccharides encountered. No

structural investigation on a purely chemical basis has been undertaken, and while it is conceded that final identification of any substance can only be completed by chemical methods, restricted investigations have been carried out on a number of the oligosaccharides encountered, the information obtained being valuable in their identification. Those investigations, which mainly concerned the effect of hydrolytic and enzymic treatments and which were carried out on a selection of the oligosaccharides, are reported below.

Quantitative Determination. The method of determination of total reducing content has already been described. To obtain the relative quantities of sugars and oligosaccharides, separation of those sugars was first effected on paper chromatograms. Chromatograms were prepared on paper 11.5 cm. wide by applying spots of the mixture 1.5 cm. from each side of the paper and between those, a continuous band of the mixture approximately 5.5 cm. wide. Separation was carried out, as described previously, for a period of four days.

Strips of the chromatograms 3 cm. wide were cut from either side, and after developing with aniline oxalate, they were replaced in their original position beside the middle portion of the chromatogram. The position of the sugars, present

on the undeveloped middle portion of the chromatogram, was then marked by drawing pencil lines between corresponding spots on each strip. Sugars were eluted from those sections of paper with cold water using the capillary method of MacLeod (1951). It was usual to combine the eluates of two similar chromatograms for determination, and in this way quantities of the order of 0.3 mg. of each monosaccharide and up to 3 mg. of sucrose were obtained.

All determinations were carried out by the Somogyi micro-copper method which measures the reducing power of the sugar, and therefore required that non-reducing oligosaccharides should first be hydrolysed.

Approximately 3 ml. aqueous solution of monosaccharides was eluted from the chromatogram section, and washed with 2 ml. water into the reaction flask. To this solution which now had a volume of 5 ml., 5 ml. of Somogyi reagent were added and the reducing power of the sugar determined.

In the determination of the quantities of non-reducing oligosaccharide, some complication arises from the use of strong acid for hydrolysis, and for this reason, MacLeod (1957) has used with success, invertase for hydrolysis of sucrose and raffinose. Reliable estimations were, however, obtained in the

following manner: 3 ml. aqueous solution of the oligosaccharide were eluted from the paper, and washed into a small conical flask with 3 ml. of 2N H_2SO_4. The flask was loosely stoppered with a glass stopper and heated in a boiling water bath for thirty minutes (forty-five minutes for solutions of tetrasaccharide). Chromatographic investigation showed this treatment completely to hydrolyse the oligosaccharides to monosaccharide units. The acid solution was cooled and neutralised with 3 ml. of 2N NaOH and the final volume adjusted to 10 ml. To this solution 10 ml. Somogyi reagent were added and the determination was then carried out in the usual way. It was found that concentrations of the hydrolysed sugar solution after neutralisation to a smaller volume and the use of only 5 ml. Somogyi reagent gave results of poor reproducibility. All sugar concentrations have been reported as glucose equivalents. Slight variations occur in the reducing power of monosaccharides towards the Somogyi reagent and consequently the true concentration of monosaccharide differs slightly from the glucose equivalent; the true figure for fructose, for example, is approximately 7% higher than its glucose equivalent. On the other hand, the absolute concentration of oligosaccharidesis smaller than their glucose equivalents because on hydrolysis of the

oligosaccharides, water is combined with the sugar residues. In the range of oligosaccharides encountered here these two factors tend to cancel one another out; thus if raffinose has a glucose equivalent of 0.145 mg., then the absolute (corrected) value for raffinose becomes 0.144 mg. Corrections of this order are clearly not worth making. In any event, no precise correction factor can be applied to the fructosan series, which represents a mixture of oligosaccharides.

Only one sample of any one species was investigated except in a few cases when qualitative analysis were repeated. It was found that duplicate determinations of the relative quantities of sugars and oligosaccharides were within the limits of ±5%. One complete example of the calculation of sugar content is quoted below.

Sugar content of Agrostis seeds:

Moisture content = 12.2%

200 g. (wet wt.) seed extracted and extract made to 200 ml.

1 ml. extract ≡ 3.90 ml. 0.005 N $Na_2S_2O_3$

≡ 3.90 x 0.140 mg. reducing sugar as glucose equivalent.

- 100 g. (wet wt.) seed contains 3.90 x 0.140 x 100 mg. reducing sugar as glucose equivalent

100 g. (dry wt.) seed contains 63 mg. reducing sugar as glucose equivalent.

Sugar	Titre ml.	Ratio	Mg./100 g. seed
Stachyose	3.85	1.23	78
Raffinose	6.30	2.08	133
Sucrose	19.92	6.55	417
Glucose	1.51	0.49	31
Fructose	1.53	0.51	32
Total reducing sugar	3.04	1	63

Additional Qualitative Investigation.

Treatment of sugar extracts with Invertase. The sugar extracts of five samples of grass seeds were treated with invertase. One drop of B.D.H. invertase concentrate and one drop of acetate buffer (pH 4.6) were added to a small volume of the sugar solution, and this was left for 24 hours at 37°C. In each case, a very big increase in glucose and fructose content, in addition to the degradation of some oligosaccharide, was observed. Table 2 contains a list of those sugars, other than glucose and fructose, detected chromatographically after invertase treatment.

Table 2

Oligosaccharides detected after Invertase treatment of Sugar extracts.

Grass	Melibiose	Manninotriose	Fructosans
Bromus sterilis	-	-	++
Bromus mollis	-	-	++
Brachypodium	+	+	-
Agropyron	+	-	++
Elymus	+	-	++

\+ Sugar detected - Sugar not detected

++ higher molecular fructosans detected

The significance of those observations is considered along with other observations reported below.

Sucrose. The failure after invertase treatment to detect the ketose, non-reducing sugar with chromatographic mobility of sucrose and the high concentration of glucose and fructose produced is further confirmation of the presence of sucrose in the seeds.

Raffinose. The sugars of wild oats were separated on 3 MM paper and the oligosaccharide in the raffinose position was eluted. To a portion of this eluate was added an equal volume of 2N H_2SO_4, and the sugar was hydrolysed for one hour in a loosely

stoppered conical flask in a boiling water bath. The hydrolysate was neutralised with NaOH, the salt precipitated and after concentration the sugars were separated chromatographically on No. 1 paper using two solvent systems. The products of hydrolysis had R_f's corresponding to those of galactose, glucose and fructose. A second portion of the eluate was treated with invertase, and the products of this degradation were fructose and a reducing sugar with the same mobility as melibiose. Those observations point strongly to the identification of raffinose in wild oats. The detection of melibiose after invertase treatment of extracts of Brachypodium, Agropyron and Elymus indicated the presence of raffinose in these seeds. Similar treatment of the extracts of Bromus sterilis and Bromus mollis verified that raffinose is substantially absent from the two Bromus species.

Stachyose

The higher oligosaccharide of wild oats was found to have an R_f value indistinguishable from that of the predominant oligosaccharide detected in Stachys tubifera, and believed to be stachyose. This wild oats oligosaccharide, after separation on thick paper, was hydrolysed, as for raffinose, in N H_2SO_4 for one hour. The products of hydrolysis corresponded to galactose, glucose and fructose, and although no quantitative determination was carried out, galactose

was clearly the predominant sugar. Treatment of another portion of the oligosaccharide solution with invertase produced fructose and a reducing oligosaccharide believed to be manninotriose.

Treatment of the sugar extracts of Brachypodium seed (Table 2) produced a reducing oligosaccharide of R_f similar to that of manninotriose but this is not detected in the extracts of the two species of Bromus or Agropyron or Elymus. It seems likely therefore, that stachyose is present in Brachypodium seeds, but absent from the other four referred to.

Fructosans

Fructosans have been detected in Agropyron, in Elymus and in all species of Bromus investigated. All but the highest molecular of those oligosaccharides were readily hydrolysed by invertase, a small quantity of fructosan which remained on the starting line of the chromatograms resisting hydrolysis. Acid hydrolysis of the homologous series of fructosans of Bromus sterilis produced glucose and fructose. It has already been commented upon that raffinose is not detected in the seeds of Bromus, and the sugar with R_f value very slightly lower than that of raffinose is considered to be the gluco-trifructose member of the fructosan series.

Correlation of Structure with Chromatographic Mobility

Consideration of the chromatographic mobility of

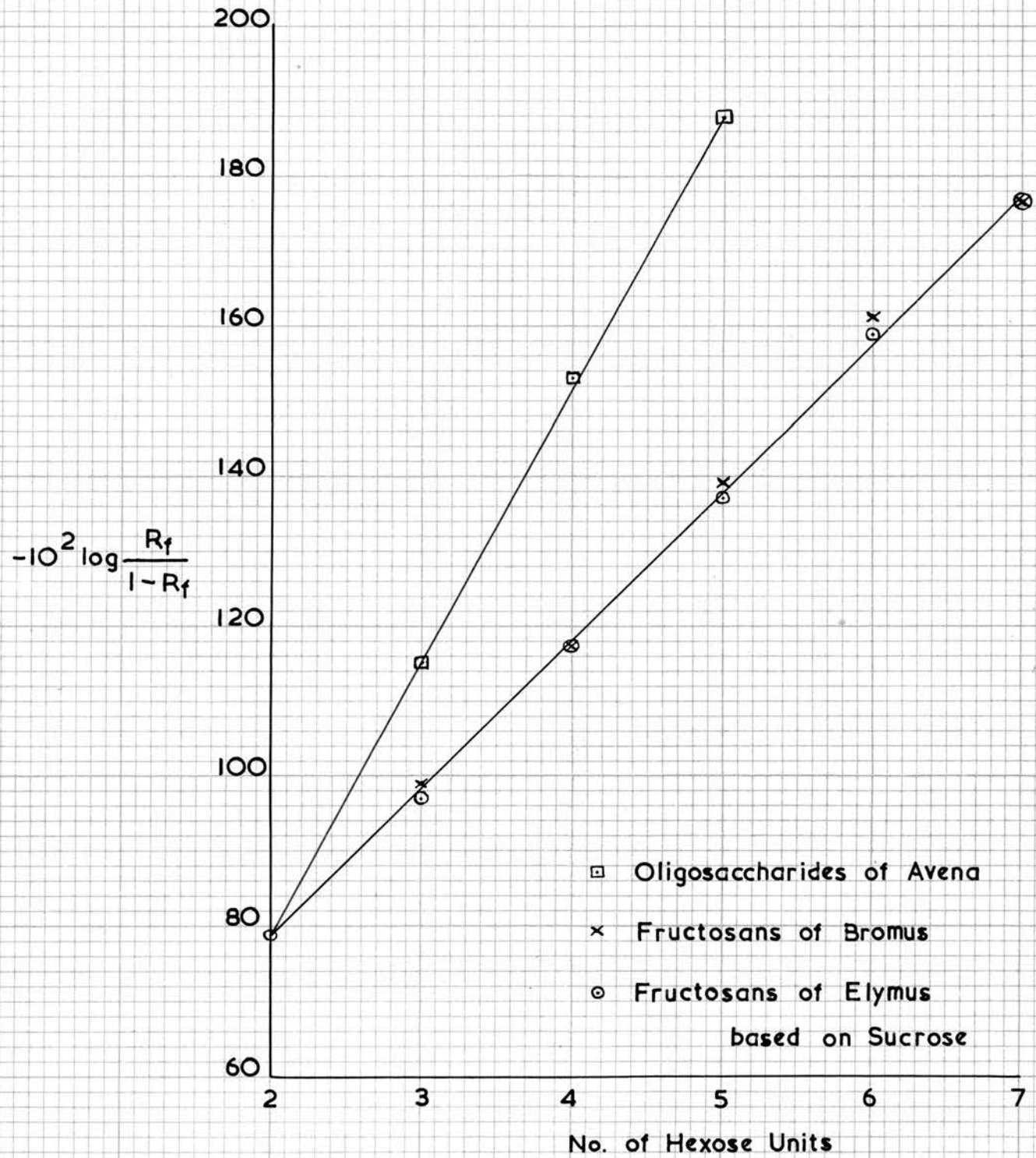
the fructosans and of the oligosaccharides associated with raffinose, provides some evidence that those two groups do consist of homologous series. French & Wild (1953) have pointed out that a linear relationship for members of a homologous series exists between $\log \frac{R_f}{1-R_f}$ and n, where n is the number of sugar units contained in the oligosaccharide members of the series.

The fructosans of Bromus sterilis and of Elymus were separated on Whatman No. 4 paper, over a period of three days, during which time sucrose of Bromus sterilis moved 33.4 cm., and sucrose of Elymus slightly farther. An R_f value of 0.14 was obtained for sucrose and the corresponding value for glucose on the same chromatogram was 0.175. The R_f value for six fructose-containing oligosaccharides in each series was calculated and the relationship obtained, on the assumption that succeeding members of the series differ by one sugar residue. Figure 1 shows that each point lies very close to a straight line, and furthermore that the same straight line was obtained for each series. This observation is regarded as evidence that those series are in fact homologous and that the oligosaccharides involved are the same in each series.

A linear relationship is also obtained for the members of the "raffinose" series, supporting the view that the oligosaccharides present are the homologues of

FIGURE 1

CORRELATION OF STRUCTURE WITH CHROMATOGRAPHIC MOBILITY.



raffinose.

Pentose oligosaccharides

Small quantities of pentose oligosaccharides have been detected on chromatograms of the alcohol extracts of cocksfoot, timothy, Agrostis and Bromus sterilis. Those pentosans, which are detected on the starting line of chromatograms, clearly occur in very small concentrations. Since this trace of pentosan of Bromus sterilis is not separated from the high members of the fructosan series, the value quoted for fructosans will be very slightly high, and the error involved is considered to be insignificant. After chromatographic separation of the sugars of Bromus sterilis, the carbohydrates on the starting line were eluted and hydrolysed in dilute H_2SO_4. The products of hydrolysis were glucose and fructose (from the fructosans) and a smaller amount of arabinose. No xylose was detected, suggesting that the pentosan was a low-molecular araban.

Raffinose isomer

A sugar with R_G 0.40 (approx.) in butanol/acetic acid/water was detected in samples of perennial ryegrass and tall fescue seeds. The use of the appropriate spray reagents showed that it was non-reducing and that it contained a ketose residue. The mobility of the sugar was distinctly higher than that of raffinose, and was almost indistinguishable from

that of glucodifructose, but no higher member of the fructosan series and, in fact, no oligosaccharide other than sucrose was detected along with it. A small amount of the sugar was separated on 3 MM paper and was hydrolysed completely in N H_2SO_4 at 90°C. for 30 minutes to produce galactose, glucose and fructose (Plate 1). Those sugars were identified by their mobility in the solvents butanol/acetic acid/water, butanol/ethanol/water and phenol/water. The oligosaccharide was very readily hydrolysed by invertase to fructose and a reducing sugar with R_G in butanol/ acetic acid/ water almost imperceptibly greater than that of maltose (Plate 2). Hydrolysis of this reducing sugar yielded galactose and glucose, identified by their mobility in the solvents already named and also in ethyl acetate/pyridine/water. In all these respects the sugars from rye-grass and tall fescue were found to be identical.

Additional investigations were carried out only on the sugar isolated from perennial rye-grass seeds. Isolation by means of a charcoal column - the technique employed was that of Whistler & Durso (1950) with slight modification in the preparation of the column. Equal quantities of activated charcoal and celite were mixed with water and poured into a glass column 3.5 cm. in diameter which was plugged loosely with glass wool at the foot. After the mixture had settled giving a

column 20 cm. high, the column was washed with a large volume of tap water, in preference to distilled water, because of its slightly higher pH, to remove the acid present in the charcoal. This method of washing differed from the method of Whistler & Durso, who washed the charcoal free of acid before preparation of the column, and this modification was successful in allowing a much faster rate of flow through the column. This was important because flow tended to decrease quite considerably while separating the sugar concentrate.

An alcoholic extract of 100 g. perennial rye-grass was concentrated to approximately 10 ml. and was added to the top of the column. After it became almost completely absorbed on the charcoal mixture, water was percolated through the column. 1 litre of water was washed through the column at a rate of approximately 200 ml./hr. This treatment was then followed by washing with 2 litres 5% ethanol, which removed sucrose, and then by 200 ml. of 10% ethanol to remove a small quantity of disaccharide sometimes remaining. Finally elution with 15% ethanol (up to 1 litre at a rate of about 120 ml./hr.) removed the oligosaccharide under consideration. Concentration of the eluate was carried out on a water-bath in the presence of a small quantity of barium carbonate. It was important to neutralise any remaining acid since

the sugar was hydrolysed quite readily by heating in water at a pH of 4. After concentration, the sugar was shown to be chromatographically pure. Non-carbohydrate impurity was removed, and a yield of approximately 150 mg. of syrup was obtained. This yield was very much lower than expected, although the quantities present in the several samples of seeds used would undoubtedly vary. Without recrystallisation, the syrup had a specific rotation of $+90.5^{\circ}$.

A sample of sugar obtained by separation on a charcoal column was treated with invertase to produce fructose and the reducing sugar. This mixture was then re-applied to a charcoal column and the monosaccharide removed by washing with water. The reducing sugar was then eluted by use of 10% ethanol.

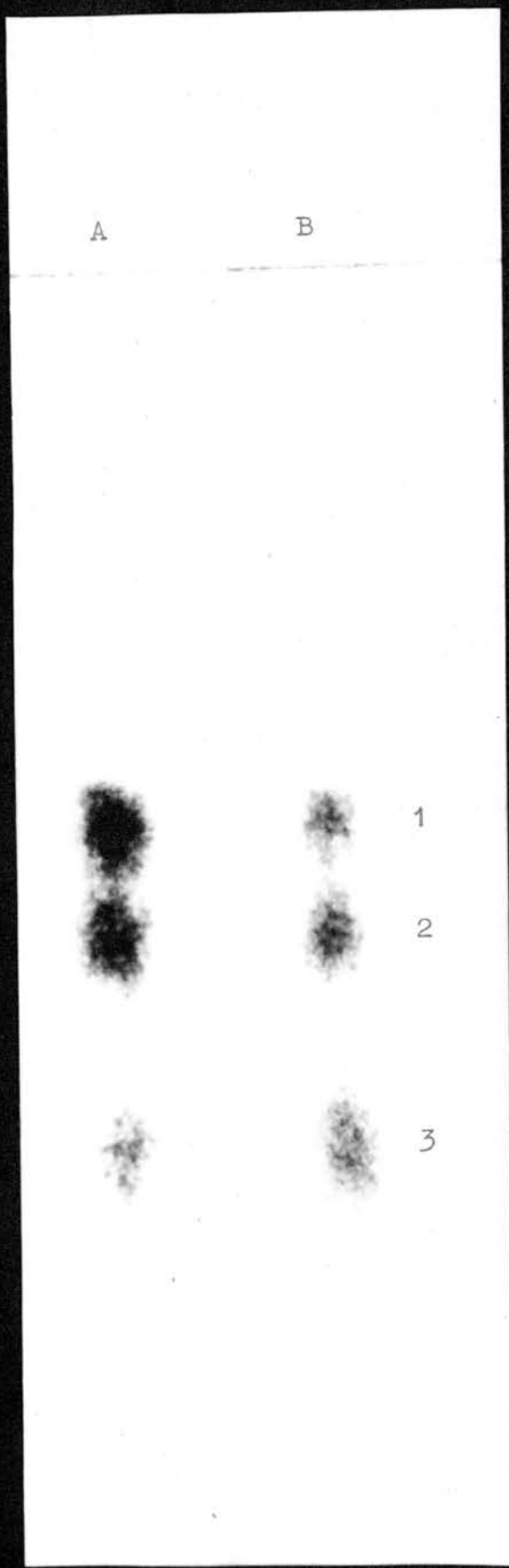
Chromatographic mobilities, and also the alcohol concentrations required for elution from a charcoal column, suggest that the non-reducing naturally-occurring sugar is a trisaccharide, and that the reducing sugar, produced as a result of invertase action, is a disaccharide. It was found impossible to estimate accurately the relative quantities of the components of the presumed trisaccharide after acid hydrolyses, in view of the inevitable partial degradation of fructose residues. The trisaccharide was accordingly treated with invertase solution and the

ratio of the reducing power of fructose to reducing oligosaccharide was found to be 1.2:1. Although a value of exactly 1:1 would ideally be expected, the value obtained suggests that the breakdown of the presumed trisaccharide produces one molecule of fructose for each molecule of reducing oligosaccharide. This oligosaccharide was hydrolysed to its monosaccharide units by use of dilute acid and the ratio of galactose to glucose determined as approximately 1:1. Electrophoretic mobility of the bisulphite complex of this sugar in sodium bisulphite solution is further evidence in favour of its being a disaccharide. It has been shown (Frahn & Mills, 1956) for a selection of disaccharides, including lactose and melibiose, that the electrophoretic mobility of a disaccharide in this solution varies from 0.69 to 0.71 times that of glucose, and more generally, that mobility of reducing oligosaccharides is a function of molecular weight. The mobility is obtained by measuring the distance between two spots on the electrophoretogram, one being the charged bisulphite-sugar complex and the other being the free sugar. With a high concentration, streaking occurs between those two spots. The sugar was subjected to this treatment in 0.4 M bisulphite for 2 hours at 600 V and 50 m.a. and an M_G value (ratio of mobility of sugar to that of glucose) of 0.68 was obtained for this sugar. An

identical M_G value was obtained for the disaccharide, cellobiose, run as a control (Plate 3). Electrophoresis was carried out on an apparatus of the design of Foster (1952).

It was determined by means of iodine oxidation (Barker et al. 1955) that the galactose residue of the reducing disaccharide is at the non-reducing end of the molecule. To approximately 10 mg. of the disaccharide, 1 ml. 0.1 N iodine solution and 2 ml. bicarbonate buffer (pH 8.6) were added at room temperature, and the volume was made to 8 ml. After oxidation, excess iodine was destroyed by addition of $Na_2S_2O_3$. The aldobionic acid was then hydrolysed in N H_2SO_4 in a boiling water bath for 30 min., and after neutralisation the products were examined chromatographically. The solution was desalted by addition of 4 vols. ethanol, or, more successfully, by first adding silver sulphate to the neutral solution to precipitate the iodide and then by addition of ethanol. After oxidising for 3 hours, galactose was detected chromatographically in large quantity, along with a small quantity of glucose. After oxidising for 24 hours, galactose and no glucose was detected (Plate 4). Glucose was therefore present as the reducing moiety of the disaccharide. A control experiment carried out on lactose produced the same result.

PLATE I


A
B
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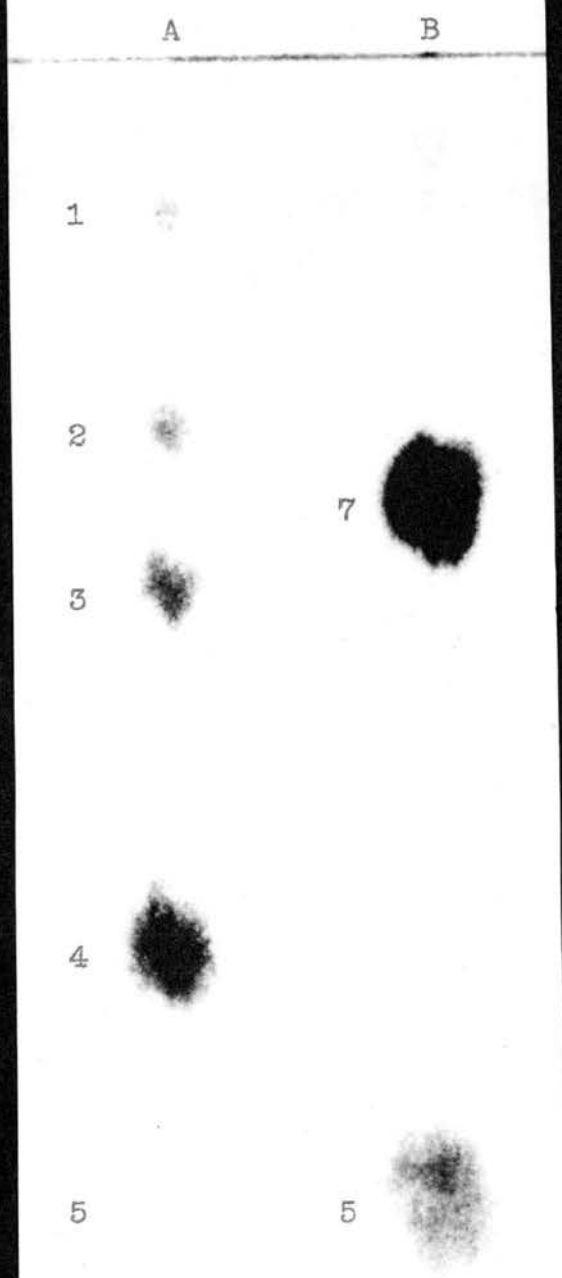
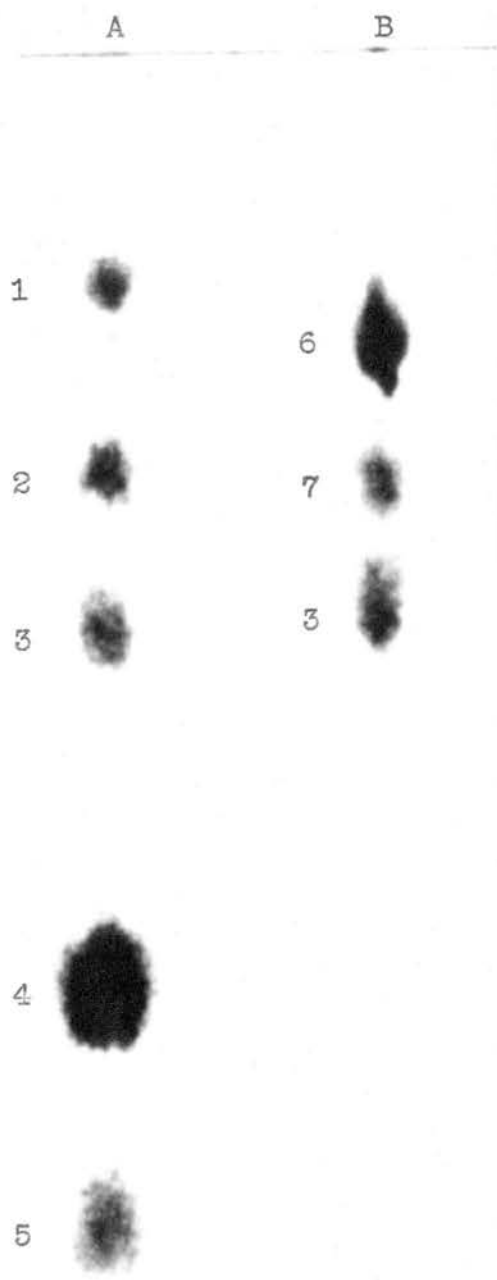
2
3

PLATE II


A
B
1
2
3
4
5
7


A
B
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2
3
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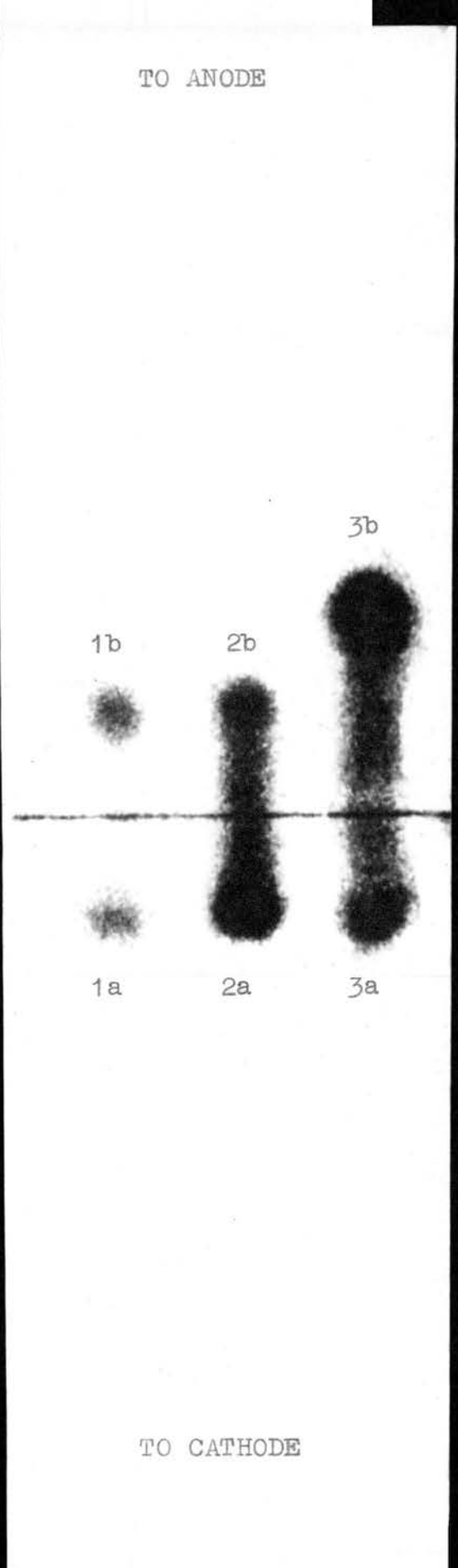
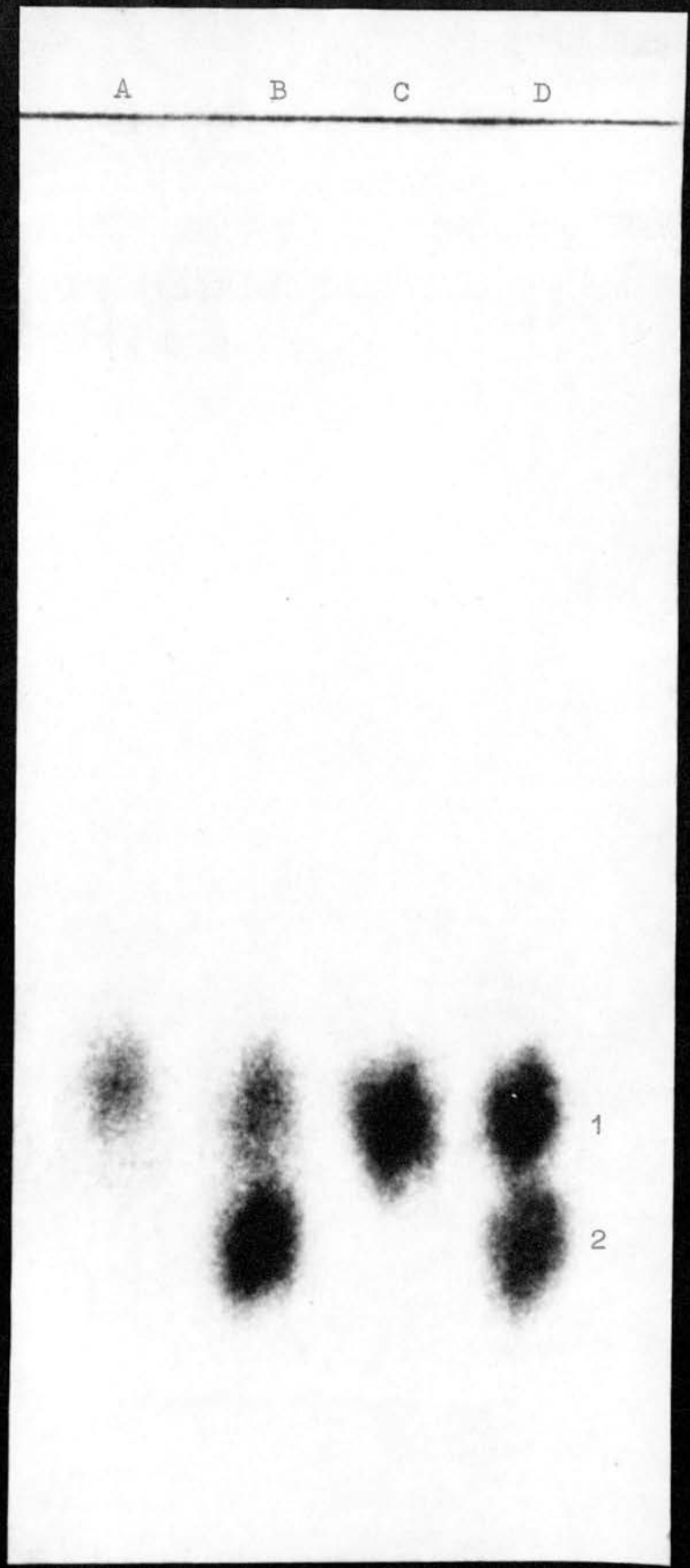
5
6
7

PLATE III


TO ANODE
3b
1b
2b
1a
2a
3a
TO CATHODE


TO ANODE
1
2
3
4
TO CATHODE

PLATE IV


A
B
C
D
1
2

Emulsin did not break down the trisaccharide so that no information with regard to its structure could be obtained by this method. A fortuitous degradation caused by bacterial action, however, produced two disaccharides, one being the reducing sugar formed by invertase action and the other being a ketose sugar with the same mobility as sucrose (Plate 2). None of the monosaccharide units, however, could be detected. The presence of a terminal β-fructofuranoside residue is indicated because of the degradation of the molecule by invertase, and ease of hydrolysis in very dilute acid.

In summary, the evidence is sufficient to state definitely that the non-reducing sugar is a trisaccharide and that the sugar produced by its partial degradation is a disaccharide. The electrophoretic mobility in borate solution of these two oligosaccharides has been obtained. Electrophoresis was carried out in 0.2M sodium borate at pH 10 for 3 hours at 1000 V and a final current of 10 m.a. The mobility of the trisaccharide is slightly higher than that of raffinose; and the mobility of the disaccharide is high, being greater than laminaribiose and only slightly lower than that of melibiose (Plate 3). Foster (1953) has observed that the mobility of oligosaccharides in borate buffer is dependent on the mode of linkage of the sugar

residues, and the general rule has been found to hold that for disaccharides, those linked in 1, 2 and 1, 4 positions have very low mobility, while those with 1, 3 and 1, 6 linkage have high mobility. There appears to be no distinction between those with α and β linkages. Melibiose and lactose, disaccharides containing a galactose residue at the non-reducing end of the molecule conform to this rule. In view of the fact that the disaccharide under consideration has been shown to have its galactose residue at the non-reducing end, it seems likely that it will conform to the general rule of mobility in borate, and would therefore contain a 1, 3 or 1, 6 linkage.

No decisive evidence of the structure of these two oligosaccharides is available by those studies but a number of indications are obtained. The trisaccharide appears to contain a terminal β-fructofuranosidic group attached to a glucose residue, as in sucrose, with a galactose residue at the other end of the molecule. It seems therefore to be similar to raffinose. Its high positive specific rotation suggests that the linkage between the galactose and glucose residues will be an α linkage. The reducing disaccharide produced by removing the fructose residue, is believed to have a 1, 3 or 1, 6 linkage; an α1, 6 linkage is present in melibiose, so that an α1, 3 linkage seems to be most likely. The structure

of the trisaccharide, most likely to fit the evidence available would therefore be O-α-D-galactopyranosyl-(1→3)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside. No reference in the literature to such a trisaccharide has been found so that it was impossible to compare it with an authentic sample.

RESULTS

Although sucrose was not invariably the most abundant sugar detected in seed extracts, it was always present in relatively high concentration, and overall was clearly the most important sugar encountered. Glucose and fructose were always present but usually in much smaller concentration. Next to sucrose, the trisaccharide raffinose was most frequently detected and with it there frequently occurred a tetrasaccharide, believed to be stachyose. A more complex oligosaccharide was rather infrequently found (most clearly in the extract of seeds of wild oats) which is non-reducing, and contains a ketose residue. Its small concentration prevented further investigation, but it seems likely to be verbascose, the pentasaccharide homologue of raffinose. The low-molecular series of fructosans has been detected in

Table 3

Sugars and Oligosaccharides of Grass Seeds

Grass	mg. sugar per 100 g. dry weight of seeds (glucose equivalent, after hydrolysis where necessary)					
	Glucose	Fructose	Sucrose	Trisaccharide	Stachyose	Fructosan
Bromus sterilis	38	120	735	-	-	2211
Bromus mollis	35	111	730	-	-	897
Bromus asper	64	82	380	-	-	419
Brachypodium	150	183	1380	286	220	-
Agropyron	158	170	194	72	-	274
Elymus	50	83	570	122	-	817
Glyceria	205	189	676	110	-	-
Festuca	49	67	168	324*	-	-
Lolium	147	127	472	970*	-	-
Poa	72	101	1010	130	64	-
Dactylis	55	37	600	184	75	-
Cynosurus	105	85	815	200	48	-
Arrhenatherum	220	200	375	Trace	±	-
Avena	106	94	626	170	134	-
Holcus	55	40	340	315	185	-
Anthoxanthum	86	88	245	154	19	-
Phalaris	19	26	286	81	-	-
Ammophila	280	270	885	330	Trace	-
Agrostis	31	32	417	133	78	-
Phleum	54	28	528	70	24	-
Nardus/						

Table 3 (contd.)

Grass	mg. sugar per 100 g. dry weight of seeds (glucose equivalent, after hydrolysis where necessary)					
	Glucose	Fructose	Sucrose	Trisaccharide	Stachyose	Fructosan
Nardus	198	152	978	217	-	-
Molinia	375	563	920	290	-	-
Sieglingia	69	75	1085	164	105	-
Spartina	344	286	10700	-	-	-
Setaria	31	21	318	38	Trace	-

* Raffinose isomer: other trisaccharide entries are authentic raffinose.

± Detection uncertain.

Table 3a

Sugars and Oligosaccharides of Grass Seeds

Trace Quantities Detected

Grass	Arabinose	Pentosan*	Galactose	Melibiose	Verbascose
B. Sterilis		+			
Lolium			+		
Dactylis		+			+
Cynosurus					+
Avena					+
Anthoxanthum	+			+	
Phalaris	+			+	
Agrostis	+	+		+	+
Phleum	+	+			
Setaria			+		

* Low-molecular pentosan on starting-line of chromatogram

+ Sugar detected.

Table 4*

Sugars and Oligosaccharides of Grass Seeds

(mg. sugar per 100 g. of seed)

Cereal	Glucose	Fructose	Sucrose	Raffinose	Fructosans	Maltose
Barley	107	26	908	450	1030	90
Wheat	92	57	836	331	1436	Trace
Rye	77	98	1857	419	4690	Trace
Oats	52	91	639	192	127	Trace
Maize	50	55	783	186	0	Trace

* From MacLeod & Preece (1954).

Table 5

Fructosan content of Grass Seeds

(mg. per 100 g. of seed)

Grass	Glucodifructose	Higher Fructosans	Total
B. sterilis	715	1496	2211
B. mollis	435	462	897
B. asper	254	165	419
Agropyron	120	154	274
Elymus	322	495	817
Barley	250	780	1030
Wheat	406	1030	1436
Rye	750	3940	4690
Oats	38	89	127

the seeds of a total of five different species. The trisaccharide found in seeds of perennial rye-grass and tall fescue grass, and showing close similarity to raffinose, does not appear to have been previously encountered. Several sugars have on occasion been detected in quantities too small for estimation. Those are the monosaccharides, galactose and arabinose and a reducing oligosaccharide with chromatographic mobility of melibiose. Small quantities of low-molecular pentosan have been detected on the starting line of chromatograms of the extracts from four species, while in one of those, Anthoxanthum, a trace of pentose oligosaccharide with chromatographic mobility slightly higher than sucrose, was detected.

In most cases only one sample of any species has been investigated for sugar content, and this possible limitation must be borne in mind when considering the results. Duplicate samples of several species, however, in which results were somewhat unexpected, have been investigated. Those were perennial rye-grass (different commercial samples) and Brachypodium (one sample obtained from the West of Scotland and the other from the Edinburgh district) and in each case, the sugar contents of the two samples was qualitatively the same. Two samples of Spartina grass seeds (both from the same source but from different seasons) were investigated because of their unusually high sugar

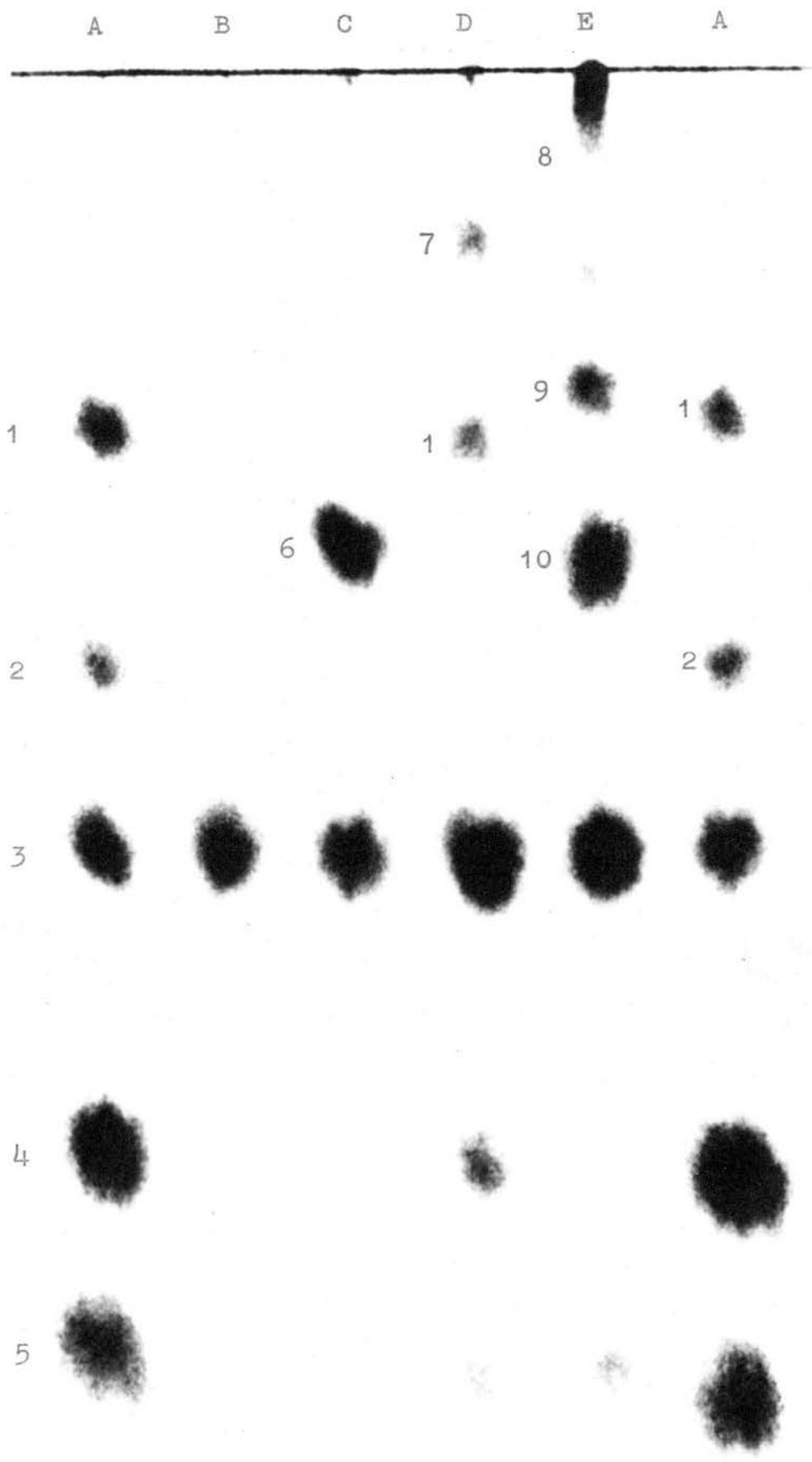
content and the results obtained were substantially the same.

DISCUSSION

A survey of the distribution of those sugars and oligosaccharides is rendered more valuable by including the data available for the sugar content of five of the common cereals. For this reason, those observations are quoted in Table 4. It will first be observed that glucose, fructose and sucrose are to be found in the seeds of all grasses examined, and that a limited number of groups of oligosaccharides are present in the seeds. Four such groups of oligosaccharides (see Plate 5) are broadly distinguished although, within those, small differences occur.

Spartina grass is the sole member of the simplest of those groups - the seeds of this grass contain no oligosaccharide in quantities sufficient for detection, and the sucrose content is extraordinarily high. It should, however, be noted that this extremely high sucrose content would tend to prevent detection of normal quantities of other oligosaccharides. With regard to the detection of sugars on paper chromatograms it should be emphasised that their detection depends

PLATE V


A
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on their relative, rather than absolute quantity in a mixture. The method is quite satisfactorily applicable on almost all occasions, allowing detection of all but those sugars which are present to an extent of less than 1% of the total sugar content. Normally this would only exclude quantities of less than 10 mg. per 100 g. seed but would make quantities of less than 100 mg. oligosaccharide per 100 g. seed in the Spartina extract difficult to detect. It is difficult to generalise about the results obtained with Spartina as this species shows no true resting stage after harvest and its "seeds" may therefore not be strictly comparable with those of the other grasses examined.

Perennial rye-grass and tall fescue grass constitute an unusually distinct group, for the seeds of those two grasses contain, in addition to glucose, fructose and sucrose, only a trisaccharide which for the sake of convenience has been called the "raffinose isomer". It is unusual that only one oligosaccharide should be found along with sucrose. There are several examples of no oligosaccharide other than raffinose occurring, but in such cases, the raffinose content is usually extremely low. Here, however, concentration of the trisaccharide exceeds by several times that of sucrose. In this respect those two grasses are quite distinct from others investigated.

A large number of the grass seeds contain raffinose along with one or two higher oligosaccharides. Those seeds which contain raffinose but no fructosans include by far the largest number of those examined, amounting to 17 out of 25 samples. In this group considerable variety in detail exists; for example, only a trace of raffinose was detected in Arrhenatherum whereas wild oats contain relatively high quantities of raffinose, stachyose and verbascose. Millet seed contains both raffinose and stachyose while Phalaris contains only raffinose. Although very noticeable differences in detail therefore exist in those sugar contents, it is nevertheless believed that fundamentally those samples are similar. Some discussion of this view follows later.

Finally the five members of the tribe Hordeae, which have now been investigated, contain low molecular fructosans which otherwise have only been detected in the Bromeae. That those fructosans should occur in such a restricted number of genera, all of which have close botanical relationship, is somewhat surprising, and the biochemical and taxonomical implications of this fact are to be discussed.

The quantities of sugar present in seeds varies quite considerably from species to species but the total content except in one case (Spartina) lies between 1-3% of the weight of the seed. The bulk of

this sugar is contained in the seed embryo and to a lesser extent in the endosperm with only traces in the husk, and since it has not always been possible to establish the proportion of seed to husk, it is impossible to regard the values quoted as absolutely comparable. Smaller seeds contain relatively less sugar than large seeds, but clearly the size of the seed does not affect sugar content per 100 g. of seed.

Monosaccharides and sucrose

Reducing sugar content varies from as little as 45 mg. per 100 g. seed to over 500 mg. with an average figure of the order of 150 mg. Except in three instances, glucose and fructose are present in similar amounts, and the considerable difference in reducing sugar content of extracts, which had been prepared under the same conditions, supports the view that no significant degradation of oligosaccharide occurs during preparation of extracts. Furthermore, seeds having high concentrations of reducing sugars represented in all cases samples which had been collected locally and air-dried in the laboratory. Although care was taken to collect only "ripe" seeds, it seems likely that the methods used to dry those samples, in some cases resulted in a certain amount of autolytic degradation. It is difficult to decide the metabolic significance, if any, of the reducing sugars, and, in view of the very small quantities on occasion

present, it is tempting to suggest that their presence is incidental to the plant metabolism. There seems to be little doubt, however, that monosaccharides can be utilised as a substrate during germination and for this reason the possibility cannot be ignored that part, at least, of the sucrose is degraded before being used for synthetic purposes. As to sucrose, it is clear that it serves as the most important sugar during germination, (see, e.g. James 1940), and in view of the fact that monosaccharide content might very well be accounted for as a result of incidental autolysis, one is inclined to the view that sucrose in fact is utilised without previous degradation. Clearly, however, the utilisation of simple sugars is a complex process, certainly involving the formation of sugar derivatives. The recent work of Ginsburg & Hazzid (1956) who have shown that labelled glucose introduced into wheat seedlings can be converted to the arabinose and xylose residues of hemicelluloses, serves to indicate the complex reactions which may occur.

Oligosaccharides

The oligosaccharides, broadly, comprise a fructosan series and a series of galactose-containing oligosaccharides. Some doubt has existed as to the function of fructosan-type molecules in plants, and although it has been suggested that they are an

intermediate in starch synthesis, it is more widely believed that they, in fact, serve as reserve carbohydrates. It may be that on occasion quantities of sucrose in excess of that required, are transformed into the fructosan oligosaccharides. Certainly during the vegetative growth of barley, conditions conducive to vigorous carbohydrate synthesis by leaves also promote the accumulation of fructosans in the internodes (Archbold, 1942); again, in "good" carbohydrate years, fructosans may be stored even in the leaves of barley and utilised - slowly - when the detached leaves are kept in the dark (Yemm, 1935).

Because of the absence of raffinose in Bromus seeds, it has been possible to calculate with some accuracy the concentration of the trisaccharide and tetrasaccharide members of the series.

Table 6

Seed	mg./100 g. seed		Ratio GF_2:GF_3
	GF_2	GF_3	
B. sterilis	715	276	2.6:1
B. asper	254	87	2.9:1
B. mollis	435	152	2.9:1

The approximately constant ratio (Table 6) of the concentration of those oligosaccharides suggests that

synthesis of higher members follows after some quantity of simpler oligosaccharides has been formed. The presence of a very high amount of the higher molecular fructosans in Bromus sterilis, however, is difficult to reconcile with this suggestion. It is difficult to assess the real significance of these fructosans, but some points with regard to their occurrence are worth considering. First is the fact that in ripe seeds, fructosans are only detected in a very restricted number of species. Furthermore, the work of de Cugnac (1931), who investigated a large number of grasses, has shown that fructosans are present in the stems and leaves of some grasses and absent from others. It is apparent that the presence of fructosans in the stems and leaves does not always result in their presence in the ripe seed of the plant. For example, de Cugnac reports that fructosans are present in samples of rye-grass and Arrhenatherum while they are not detected in their seeds. It seems possible that the carbohydrate content of the seeds is a result of synthesis within the ear and is not simply an "overflow" from the stem, a suggestion which is consistent with the findings of Porter et al (1950), that in fact surprisingly little transportation of carbohydrate takes place from the stem to the grain. The detection of the "raffinose isomer" in seeds of perennial rye-grass

and the report (Aspinall & Wylam, unpublished) of raffinose in the stem is further evidence in favour of this point of view. Since, however, strict comparison could only be made on stems and seeds of the same sample of grass, not too much reliance should be placed on this observation. It is, however, firmly established that starch is only very rarely present in the leaves and stems of grasses, though it accounts for approximately 60% of the dry matter of the grains of cereals.

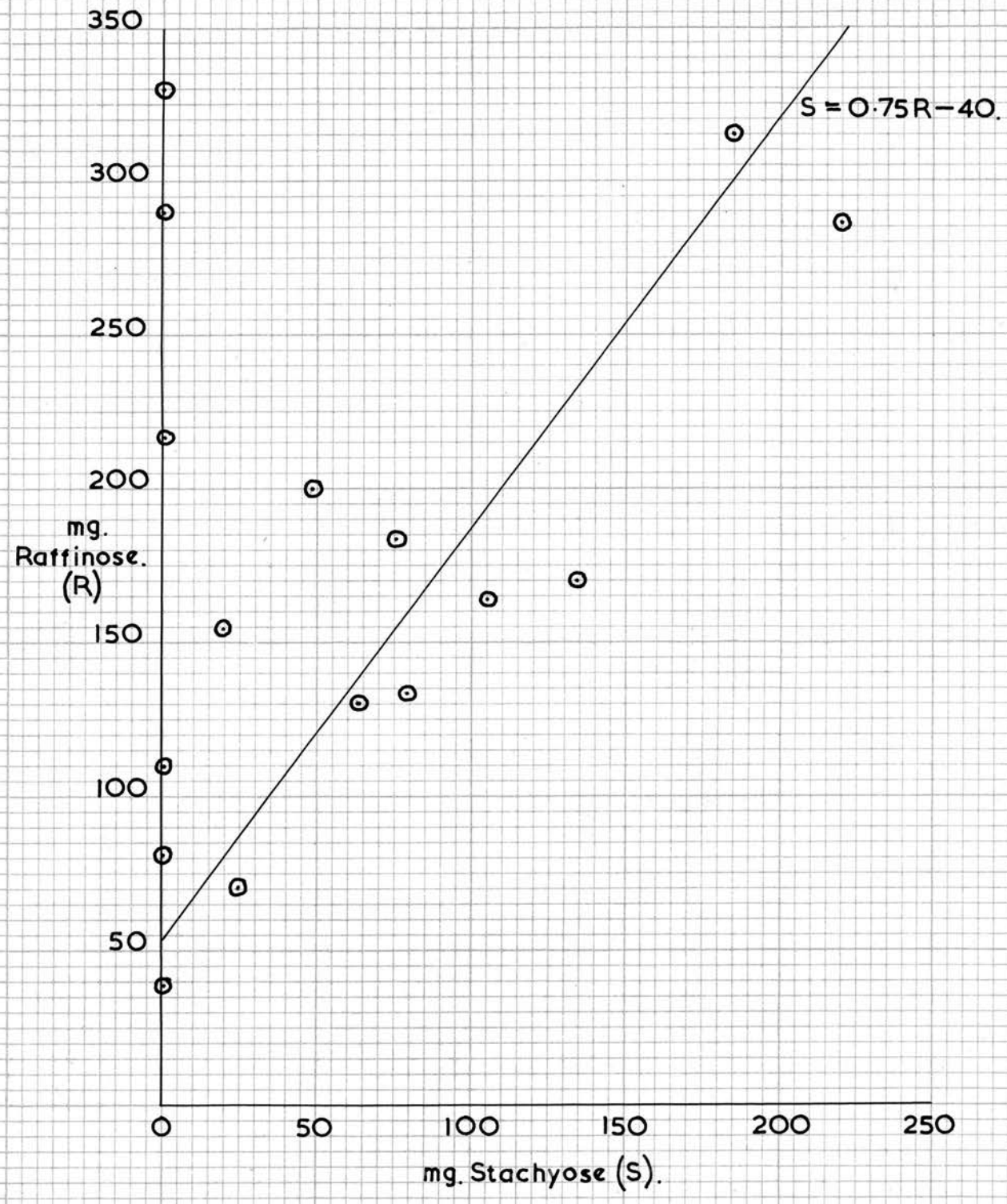
The occurrence of the raffinose isomer represents a special example of the galactose-containing oligosaccharides. It seems likely that those serve as reserve carbohydrates and this view is supported by the utilisation of raffinose in germination, and by similar observations on the raffinose isomer, reported later. It seems likely that those oligosaccharides are synthesised within the seed but clearly the mechanism of such synthesis differs considerably. For example, the raffinose isomer, which is present in quantities exceeding the sucrose content, is not accompanied by any other oligosaccharide, while raffinose most frequently occurs along with higher homologues. Some evidence is derived from the results of Table 3 in favour of the view that synthesis of stachyose follows after a minimum concentration of raffinose is reached. Stachyose and

raffinose together occur in 10 of the samples investigated and the regression equation for the line plotted in figure 2,(S = 0.75 R-40) shows the relationship between their concentrations. This suggests that while raffinose will continue to be synthesised after synthesis of stachyose commences, a certain amount of raffinose, approximately 40 mg. per 100 g. seed, will first be formed. Exceptionally, however, (e.g. in cultivated barley and Molinia) relatively high concentrations of raffinose may occur, unaccompanied by any perceptible accumulation of stachyose. Verbascose is detected too infrequently for any similar relationship to be considered for it. Furthermore, no clear quantitative relationship exists between sucrose and oligosaccharide content; the sucrose-raffinose ratio is most frequently of the order of 3:1 but in fact varies from 1.6:1 to 8.4:1.

Of the extracts in which fructosans occur, those from the Bromeae are somewhat exceptional, no raffinose being detected in them. In other cases, raffinose does occur with fructosans but the presence of stachyose is less certain. The detection of stachyose is rendered more difficult by the presence of the fructosans but only very rarely (in one sample of an Ymer barley) was there any evidence of the occurrence of both stachyose and fructosans. It seems not unreasonable to assume that given a limited

FIGURE 2.

RAFFINOSE AND STACHYOSE CONCENTRATIONS (Mg. per 100g. seed)
[See Table 3]



amount of simple carbohydrate "units" available for synthesis, the synthesis of the fructosan series would be accompanied by a restriction of the tendency for formation of the "raffinose" series.

The molecular size of oligosaccharides obtained during this investigation is restricted to a somewhat low value. While fructosans have been obtained containing as many as ten sugar residues, the highest member detected of the "raffinose" series is a pentasaccharide. Although it is true that higher members would have a low solubility in 80% alcohol, nevertheless, the complete failure to detect even a trace of any such oligosaccharides, coupled with the fact that higher molecular fructosans are present, is good evidence of the absence of any. It is confidently believed that all low molecular oligosaccharides are extracted.

General

It has been shown that the sugars and oligosaccharides occurring in the seeds of the grasses investigated place themselves into four broad groups, and further, it has been suggested that such variations are the direct result of different systems - presumably enzymic - functioning during ripening and possibly during post-harvest treatment. Any external factors which might account for such differences must however be taken into account. It is well known that

environmental differences and differences in nutritional treatment radically affect the carbohydrate content of the stems and leaves of the plants. It is of first importance to the present investigation, however, to observe that Archbold (1938) detects as little as an 11% increase in carbohydrate content of the ear of plants grown under conditions of nitrogen deficiency in comparison to a tenfold increase in the stems. This is in accord with the well-established fact that environmental conditions exert a much smaller effect on the reproductive parts of flowering plants than they do on the vegetative parts. The regularity apparent in the numbers, size and arrangement of the various flower parts is reflected in the universal use of floral characteristics in plant classification, and it would appear that the biochemical composition of the reproductive structures (e.g. seeds) may show a consistency similar to that taken for granted in the external morphology of these structures.

It certainly seems likely then that any nutritional differences would fail to account for anything but a small quantitative variation. A more serious objection arises from the possibility of obtaining samples at a stage other than absolute ripeness. With regard to this problem, it should first be emphasised that seeds were collected, as far

as could be judged, at ripeness. Moreover, since the work of Harris & MacWilliam (1954 a) shows that in barley, carbohydrate differences over the last four weeks of ripening are rather restricted, it seems likely that very little difference would be expected by harvesting seeds a few days before absolute ripeness. The more complex possibility presents itself that the state judged to be ripeness may not represent the same "resting-point" in the metabolic process of ripening and germination in every grass. In view of the changes in carbohydrate content which do occur during the process of ripening and more particularly of germination, there does seem to be a real possibility of accounting for some of the differences in carbohydrate content on the assumption that "ripeness" is a stage in the metabolism of the plant which varies somewhat from species to species. If in fact this is the case, the carbohydrate differences observed would not be rendered irrelevant, but would serve to illustrate that the balance of enzymes responsible for the formation of the seed will differ in different species. Although this explanation would undoubtedly account to some extent for carbohydrate variations, it seems certain that it does not account for the more distinct of qualitative differences. For example the presence of a large quantity of raffinose isomer in the absence of any other oligosaccharide

seems very unlikely to represent one stage in a metabolic sequence leading to - or from - the production of quantities of raffinose and similar oligosaccharides. The evidence, is strongly in favour of the view that distinct enzymic systems are responsible for the formation of those differing oligosaccharide contents.

It is suggested, therefore, that two explanations may be offered to account for sugar and oligosaccharide differences in the ripe seeds; first, that ripeness in fact represents a different stage in different plants; and second, that quite distinct enzyme systems are involved. It is emphasised that even the first possibility would reflect some variation in metabolic processes, but the clear-cut distinctions involved in the second possibility are more strongly favoured, at least for some of the oligosaccharides involved.

Finally, in view of the restricted number of groups into which the grasses are divided by virtue of the sugar content of their seeds, consideration is given to the possible application of those observations to the taxonomy of grasses. This question is discussed in Section III.

SECTION II

WATER-SOLUBLE POLYSACCHARIDES

INTRODUCTION

Some reference has already been made to general polysaccharide distribution in plants, and to the molecular entities involved in certain plant functions. Two broad distinctions - reserve food and structural polysaccharides - are observed, and it is a tempting hypothesis, partly substantiated by the apparent inertness of cellulose, that those groups are completely distinct and self-containing. It is, however, a widely-accepted opinion that such absolute division is unwarranted and an increasing volume of experimental evidence, still insufficient and tending to be circumstantial, points to the participation of hemicellulosic material in an other than purely structural role.

Hemicellulose is a term suggested by Schulze in 1891 to cover a group of polysaccharides obtained from plant material by extraction with dilute alkali. His reason for using this name - that those polysaccharides were chemically related to cellulose - has proved incorrect, yet some justification remains in the fact that most of this material is situated in close association with cellulose in the plant. As used today, the term "Hemicellulose" enjoys some licence in meaning. Its most widely accepted meaning includes those polysaccharides which are extracted by soda from a plant after previous aqueous and ammonium oxalate

extraction. This definition of hemicellulose, excludes not only cellulose but also starches, pectins and mucilages and other polysaccharides extractable by water. With regard to these water-soluble polysaccharides, evidence is accumulating to show that some of them bear a close chemical similarity to the so-called hemicellulose. It is thought that hemicellulose found in lignified tissue exists in chemical association with lignin, separation from which presents considerable difficulty. Of more general terms in use, holocellulose represents the mixture of cellulose and hemicellulose after removal of lignin.

A vigorous onslaught on hemicellulose chemistry has largely involved investigation of the carbohydrates obtained in the first place from wood, and later from grasses. With the exception of certain early investigations, including that of Tollens & Stone (1888) who prepared a hemicellulose from brewers' grain, sustained investigation started about the year 1920. O'Dwyer in 1923 investigated a preparation from American white oak, showing the presence of residues of xylose, arabinose, mannose and galactose. Such and other investigations continued, demonstrating that the hemicellulose of wood was substantially a xylan polysaccharide, while investigation (Buston, 1934) of similarly extracted material from cocksfoot grass showed it to be a galacto-araban. The group of

mannose polysaccharides which may contain galactose or glucose residues as substituents, presents interesting information on the probable utilisation of hemicellulosic material both for structural purposes and as a food reserve. Mannan which is the main constituent of the cell-wall of palm seeds disappears on germination and is believed to be utilised as a food.

The recent development of paper chromatographic methods has provided further impetus to those studies, allowing, as it does, the relatively simple identification of sugar residues, and furthermore increasing very greatly the value of the methylation technique in structural studies.

A series of investigations by Aspinall et al. (1954, 1956, 1956) carried out on xylans of woods and grasses, shows that their molecular structure almost invariably consists of a basic structure of 50-80 xylose residues joined in a chain by β 1-4 linkages, but that a wide variety exists in the nature of the small side-chains and in the position and nature of minor substituents, which most frequently consist of arabinose and uronic acid residues. It is of interest to record that, throughout hemicellulosic investigation, there is a tendency for preparations to contain predominantly either galactose and arabinose residues or xylose and glucose. Those two groupings are by no

means invariable but are nevertheless frequently encountered.

In general terms, the source of interest in such polysaccharide investigations, in the years following 1920, resolved itself into three parts. The first was the development of extractional methods, frequently requiring pretreatment of the plant, which would provide carbohydrate free from non-carbohydrate contamination, and yet as little degraded as possible. The second, has been the search for satisfactory means of fractionating the heterogeneous mixture of polysaccharide almost inevitably obtained by the extractional methods available. The value of structural investigations is seriously lessened if previous fractionation is not satisfactory and the urgent need for improvement in fractionation methods has recently been emphasised by Hirst (1955). The third part of those investigations has consisted of structural studies on the preparations. Those studies have most frequently made use of chemical investigation mainly by application of the methylation technique and, to a lesser extent, periodate oxidation; further information has been derived from enzymic studies, and it seems likely that more use will be made of this method in the future.

Precipitation by Fehling's solution has frequently been applied in attempts at carbohydrate

fractionation and it seems likely that the limited success which this technique affords, depends on a chemical basis. It is usually necessary, however, to resort to separations obtained by preferential precipitation on addition of precipitants, most frequently alcohol or acetone. The fractionation method of Norris & Preece (1930) was developed at an early stage to exploit to maximum advantage both Fehling's solution treatment and preferential precipitation. In essence, it consists of neutralisation of the soda extract, addition of two half-volumes of acetone and removal of any precipitate at each stage; further separation being effected by redissolving each precipitate and the subsequent addition of Fehling's solution, followed by acetone to each. Clearly this scheme is mainly based on empiricism, but in the absence of more selective methods, it has served its purpose with success.

The polysaccharide of the seed of the Gramineae, with the exception of the cereals and in particular barley, have attracted almost no attention. Starch in those cereals has naturally been studied, but at a very early date interest was aroused in a substantially non-starchy product obtained by water extraction of the ground seed of barley. The material, comprising about 2% of the weight of the seed, first obtained by O'Sullivan (1882), was extracted

in two fractions, first by cold water and then by aqueous extraction at 40°C. On the assumption that those preparations held a close chemical relationship to starch, they were called respectively β- and α-amylan. This assumption has proved largely mistaken, but almost inevitable, in view of the analytical methods available to the investigator. Sporadic investigation on this subject occurred later - by Lindet (1903) and Brown et al. (1907) as a result of which the cold water extract was shown to contain a material of high negative rotation which was substantially pentosan in nature. It was therefore established that β -amylan, at least, had no starchy properties. It was not till 1938 that any further significant development was made when Piratsky & Wiecha (1938) obtained a preparation corresponding to O'Sullivan's α-amylan, which they found to consist entirely of glucosan. Those preparations became known as "barley gums" - the name denoting the high viscosity of aqueous solution of the preparations. In this way they resemble the plant gums, to which, however, they show only slight structural similarity.

The effect of autolytic systems during extraction is to alter yields of the preparations and although rather small variation is observed in the qualitative composition of gums, very considerable quantitative difference may occur. It is for this

reason that only limited significance may be attached to earlier work, in which satisfactory inactivation of the enzyme systems was not usually ensured. The problem of complete enzymic inactivation and the extraction of gum material in its undegraded natural state has recently received more attention, and treatment with boiling alcohol is frequently practised. Meredith, Watts & Anderson (1953) consider that refluxing in alcohol followed by papain extraction is the most satisfactory of inactivation treatments, resulting in extraction of the gum in a condition nearest its natural state.

Recent interest in those somewhat unusual polysaccharide materials has led to a considerable clarification of their nature and function. Preece, Ashworth & Hunter (1950) by application of the Norris & Preece fractionation method obtained two fractions, corresponding to the α- and β-amylan of O'Sullivan, the first being substantially hexosan, the other containing almost equal amounts of hexose and pentose residues and following this observation, an infinitely more valuable, if somewhat lengthy fractionation method was developed (Preece & Mackenzie, 1952a). After water-extraction at 40°C. and concentration of the solution, increasing quantities of ammonium sulphate were added, producing precipitation at certain stages; further fractionation was achieved by

further treatment of the products with the salt. The spectacular effect of this mild treatment was to produce from barley gum extract a pure laevorotatory glucosan, conveniently termed β-glucosan. Further smaller fractions rich in pentosan were also obtained. Little is known of the mechanism of this fractionation, but its success and its advantage over the more usual Fehling's solution treatment, where molecular degradation might easily occur, distinguish it as an outstanding contribution to polysaccharide investigation. Structural investigation (Aspinall & Telfer, 1954) indicates the β-glucosan component of barley gum to be a linear molecule with molecular weight of the order of 20,000, and having an approximately equal number of β1.4 and β1.3 linkages. The complete distinction of this molecule from starch and cellulose was therefore established.

The widespread occurrence of cellulose and starch in the plant kingdom ensures that polymers of glucose are the most frequently encountered of plant polysaccharides. It is, however, true that with the exclusion of those two molecular species, glucose in the polymeric form is rather infrequently encountered, and, for this reason, particular interest is attached to the finding with respect to the glucosan of barley. Small quantities of glucosan are detected in hemicellulosic preparations, but those are most

frequently present as substituents of a xylan molecule. Laminarin, which is present in the sea-weed laminaria contains polymeric glucose while lichenin is a constituent of Iceland moss. Of most significance to cereal chemistry is the report (Morris 1942) of a lichenin-type molecule in the seeds of oats. Chemical investigation has shown laminarin to contain glucose residues joined by β1.3 linkage while lichenin from Icelandic moss (Meyer & Gurtler, 1947) is reported to consist of glucose residues with approximately 70% β1.4 and 30% β1.3 linkage. It appears, therefore, that barley β-glucosan differs from lichenin only in its ratio of β1.4 and β1.3 linkages. Values reported for specific rotation are -12° for barley β-glucosan, -10° for laminarin (both in water) and +8.3° (in sodium hydroxide) for lichenin. Comparison of those polysaccharides with nigeran, which is an α-linked glucosan is of interest. This bacterially synthesised polysaccharide has been intensively investigated by Barker, Bourne & Stacey (1953) and like barley β-glucosan, is found to contain a mixture of 1.4 and 1.3 linkages.

Following a survey of the unfractionated gum preparations obtained from five common cereals (Preece & Mackenzie, 1952b) a survey was undertaken of the fractions obtained by ammonium sulphate treatment of aqueous extracts of those cereals - barley, wheat, rye,

oats and maize (Preece & Hobkirk, 1953). A preparation similar to the β-glucosan of barley was obtained from oats, presumably corresponding to the lichenin of oats reported by Morris. Both wheat and rye were found to be relatively rich in pentosan content, to the exclusion of β-glucosan - the pentosan being an arabo-xylan molecule and having a very high negative rotation. The structural investigation of Perlin (1951) on water-soluble pentosan of wheat showed it to consist of a xylan chain with arabinose residues as side-chains. Perlin considered the water-solubility of the molecule to depend on the number of arabinose substituents, bearing in mind the fact that such molecules may be rendered insoluble by removal of arabinose residues. The work of Preece & Hobkirk supports Perlin's view that water-solubility of the wheat pentosan is increased by increase in number of arabinose residues, but it would appear from their work that such a relationship does not hold for the pentosan of rye. The general conclusion of this comparative survey of the cereals was one of considerable contrast in the contents of water-soluble polysaccharides, both in composition and amount, yields varying from 0.4% in maize to over 1.5% in barley.

The cereal gums - the term used to include those water-soluble polysaccharides obtained from the seeds of cereals - are shown then to include a β-glucosan

and an arabo-xylan in addition to quantities of other molecular species. By their physical properties both β-glucosan and arabo-xylan do in fact deserve to be included in the term "gum" but certain of the other water-soluble constituents are quite non-viscous in solution. Varying amounts of glucosan, starchy in nature, are neither "gummy" nor presumably are they included in those polysaccharides which are situated in the plant cell wall to which the gums are believed to belong.

Very little is known regarding small amounts of other materials detected. Mannan, in trace, has been reported from barley, while galactan is generally obtained from that fraction of the water-extract not precipitated by ammonium sulphate. This fraction is further characterised by high araban content. Paper electrophoretic separation of certain of those more soluble fractions has been effected (Preece & Hobkirk, 1955), and those separations although not resolving each molecular species, point to the presence of arabinose residues in combination both with xylose and galactose and possibly as free araban. Although fructosan, other than low molecular fructosan soluble in 80% alcohol, has been reported present in barley and oats (Harris & MacWilliam, 1954a and Anderson & Greenwood, 1955) it appears to represent a very small part of the polysaccharide mixture. Of

significance is the complete failure to detect any but the smallest amount of uronic acid residues in those water-soluble gums. It seems that what is generally regarded as pectic material is substantially absent from cereal extracts, although the detection of arabinose and galactose residues indicates the possibility of the presence of the polysaccharides so commonly associated with pectin. Further, it is probable that extraction at 40°C. would not permit complete extraction of pectic material.

Meredith & Anderson (1955) have discussed the possibility of molecular combination between the major components of barley gum. It is acknowledged by them that the separation of glucose from pentosan by the mild ammonium sulphate treatment lends support to the view that those are distinct molecules. They, however, put forward an argument in support of the possibility of combination. The significance of nitrogen content in barley gum is also discussed by Meredith, Watts & Anderson (1953). They report as little as 0.1% nitrogen in the purest samples, and consider that this may be contained in molecular complex although Preece & Hobkirk (1953) have reported the virtual absence of nitrogen from preparations obtained by ammonium sulphate fractionation.

Recent investigations (Preece & Hobkirk, 1954) reinforces the view that the cereal gums bear close

chemical and biochemical similarity to certain, at least, of the cereal hemicelluloses. Soda extraction yielded both glucosan and pentosan, with physical properties similar to those of the gums, the only point of striking distinction, excluding solubility differences, being a higher, albeit still low, uronic acid content. It is observed that the uronic acid residues are situated in the husk and are believed to be contained in the xylan molecule. This observation serves to introduce the question of the distribution of those polysaccharides throughout the grain. It has been established that the soda-soluble pentosan is present both in husk and endosperm; the husk-type being typified by containing uronic acid residues and therefore bearing greater similarity to certain wood and grass hemicelluloses. Enders et al. (1938) have estimated that one-sixth part of the water-soluble pentosan of wheat is situated in the husk; five-sixths being found in the endosperm. As to barley gum, it has been shown to be almost completely situated in the endosperm.

Some information has been obtained (Preece & Mackenzie, 1952a; Preece & Hoggan, 1957) regarding the behaviour of cereal gums and hemicelluloses during germination in a brewery malting. It is shown that water-soluble β-glucosan disappears and the initially insoluble soda-soluble material is reduced to a very

small quantity. Studies of the modification of barley during malting had been undertaken by Brown & Morris as early as 1890, who observed that the endospermic cell-walls disappeared during germination. Later observations (Grüss, 1896) were in substantial agreement, though inclined to the view that the breakdown of the walls was not absolutely complete. The belief that gum-like material contributes to these cell-walls is therefore supported by strong circumstantial evidence. During malting, changes in pentosan content are less spectacular though a restricted amount of solubilisation and degradation occurs.

The picture emerging is of a water-soluble polysaccharide mixture present in the seeds of the cereals, which clearly displays close similarity to hemicellulose material also present. The view might be supported, with some reason, that the term "water-soluble hemicellulose" would better be substituted for "gum". Preece (1957) suggests that those polysaccharides of the barley grain consist of the following groups:

i) Hemicellulose of purely structural significance; insoluble in water

ii) Hemicellulose, initially water-insoluble, but utilised on germination

iii) Water-soluble hemicelluloses - the gums.

The striking changes which occur during germination are presumed to reflect the presence of enzyme systems capable of attacking the cereal gums and the isolation of pure β -glucosan and arabo-xylan and their availability as natural substrates has considerably increased the value of studies on the cereal enzymes. General agreement has been reached on the enzyme systems degrading the β -glucosan of barley. A cytoclastic enzyme is believed to exist which attacks the interior of the molecule while a cytolytic enzyme degrades the molecule by attacking it at the extremity of the chain. In addition to those enzymes, Preece, Aitken & Dick (1954) report evidence of a cellobiase degrading the cellobiose, produced as an intermediate product. Preece & Hoggan (1956) have demonstrated the enormous increase in glucosanase activity which occurs during malting, and an increase in the much less active pentosanase system is also observed (Preece & MacDougall, unpublished).

Clearly little advance could be made in an understanding of the gum-like polysaccharides by investigation in only one direction. A combination of extraction and fractionation studies, chemical structure and biochemical behaviour gradually has allowed an understanding, still imperfect, of the part which they play in the vegetable kingdom. That they constitute a small proportion of the seed by weight,

is considered no deterrent to their investigation, for sufficient is already understood to show that they are vitally concerned with the structure of the seed and the mechanics of its germination.

EXPERIMENTAL

Previous investigation of the water-soluble polysaccharides of the seeds of cereals (Preece & Mackenzie, 1952b; Preece & Hobkirk, 1953) has shown distinct variation in the quantity and nature of products obtained. It was, therefore, decided to investigate similarly a relatively large selection of members of the Gramineae with a view to establishing what molecular entities were present and to make a comparative survey of those preparations. In view of this intention, standard methods of extraction and precipitation were in the first place used. While precipitation by means of Fehling's solution and acetone was considered not entirely unobjectionable, since some degradation of polysaccharides occurs (Hobkirk, 1955) it was nevertheless regarded as the most reliable reagent for a comparative survey of this kind, as the products obtained are virtually pure carbohydrate.

Preparation of aqueous extracts

Quantities of seeds, depending on amounts available, but usually 200 g. were ground fairly finely in a coffee mill and then treated with 80% ethanol, as described previously for extraction of sugars. In addition to extracting sugars, this treatment is intended to inactivate enzymes completely, and furthermore to extract some protein and other

vegetable matter. After four treatments under reflux, each of thirty minutes duration, the entire mixture, while still hot, was transferred to a Buchner funnel and as much alcohol as possible removed from the grain. The grain was then spread on a glass plate and dried at room temperature overnight.

Aqueous extraction was carried out by adding water, previously heated to 40°C., amounting to as much as four times the weight of the seed. The mixture was stirred continuously for thirty minutes, the temperature being maintained at 40°C. in a water-bath. The supernatant liquid was then decanted through muslin, previously boiled to remove starchy material, and the filtrate centrifuged at 3000 r.p.m. for from ten to twenty minutes, depending on the viscosity of the solution. After returning all solid material to the extraction vessel, a suitable volume of water was added and further extraction carried out. In this way four extractions of thirty minutes duration were carried out, the ground grain being squeezed as dry as possible in the muslin after the final extraction. The aqueous extracts were combined - a volume of approximately two litres resulting from extraction of 200 g. seed - and filtered through a Buchner funnel, containing two filter papers covered by a pad of kieselguhr. This pad was prepared by pouring an aqueous suspension of kieselguhr onto the

filter paper. Filtration of the extract was continued until it was obtained water-bright, sometimes as many as four or five filtrations, with renewal of kieselguhr, being necessary. It was noticeable that although one filtration sufficed to render some extracts water-bright, others still retained slight opacity, after five filtrations. Testing with iodine suggested that in general, one or two filtrations sufficed to remove suspended starch grains, and it is thought that any material remaining would be proteinaceous in nature.

The bright extract was sterilized by boiling immediately after filtration. It was then concentrated in a porcelain basin on a water-bath to a small volume suitable for precipitation, an extract from 200 g. seed being concentrated to 150 ml. and to correspondingly smaller volumes for smaller extractions. Some coagulation invariably occurred on concentration of those extracts which had presented difficulty in rendering clear, and in those cases, the solution was again filtered at an intermediate stage of concentration. A tendency for the formation of a skin on the surface of the extract was observed in a number of the more viscous solutions and since previous workers had shown such skins to consist of gum material, it was not possible to practice filtration of those solutions after concentration. Such a step,

however, did not on any occasion seem necessary since the more viscous solutions had invariably filtered water-bright quite readily.

Precipitation of gums

The gums were precipitated by use of Fehling's solution and acetone (Preece, Ashworth & Hunter, 1950). To the aqueous concentrate, kept at approximately 10°C. was added one half-volume of Fehling's solution No. 2 and mixed thoroughly until all solid was dissolved. One half-volume of Fehling's solution No. 1 was then added and the solution mixed thoroughly. In several instances precipitation occurred at this stage and this precipitate was removed either by squeezing through muslin or by centrifugation. Certain observations are made later with regard to the nature of precipitation obtained by the use of Fehling's solution and acetone and to the unusual precipitation of water-soluble polysaccharides by use of Fehling's solution alone. After removal of this precipitate, or if no precipitation occurred, acetone amounting to 40% of the volume of the gum solution, was added. The resultant precipitate was removed by centrifugation and dissolved in a small volume of N.HCl, again at 10°C. Any precipitate, obtained with Fehling's solution alone, was similarly dissolved. The carbohydrates were reprecipitated by adding acetone to a concentration of 60%. The preparation was washed with three 100 ml.

portions of 60% aqueous acetone, the first of those containing 20 ml. N.HCl, and finally with three portions of 95% ethanol. The precipitate was then transferred to a sintered glass crucible and allowed to dry in vacuo overnight. It was then dried at 100°C. for three hours, weighed, ground in a mortar and put in a tightly stoppered glass tube. It is important to pay meticulous attention to the treatment following initial precipitation of the gum; time must be taken to break down all solid material in the HCl so that the copper salts are completely dissolved; each washing with aqueous acetone, which removes traces of salt and some colouring matter was prolonged for thirty minutes during which time the carbohydrate was mixed completely and lumps broken down as completely as possible. Centrifugation between each washing was carried out at only 1000 r.p.m. to prevent the precipitate becoming too tightly packed.

Properties and composition of preparations

The preparations were white or off-white in colour and in appearance varied from quite powdery to fibrous. All redissolved in water by stirring at about 80°C. and gave bluish coloration with iodine. Specific viscosity of 0.5% aqueous solutions were determined by means of an Ostwald Viscometer (time of flow for water about 25 seconds) in a thermostatically-controlled water-bath at a temperature of 25°C. Where possible,

specific rotations were also obtained, in 0.5% aqueous solution in a 2 dm. or 1 dm. tube at room temperature (about 18°C.). The accurate determination of specific rotation of those preparations was a matter of difficulty and with many of the preparations accuracy of better than ± 5° could not be claimed. The practice was employed of determining as accurately as possible the reading on the polarimeter and subsequently filtering the solution through a very thin pad of kieselguhr. A very much more sensitive reading (usually ± 3°) could then be obtained and it is clear that no marked change in rotation is effected by this filtration, since the value obtained was always intermediate between the limits of the reading obtained before filtration. Furthermore, the solution was in contact with the kieselguhr pad for only the briefest of time. Details of yields, viscosity and specific rotations are reported in Table 7.

The approximate purity of those preparations was determined by measuring the reducing power of a known quantity after hydrolysis. After determination of the relative amount of hexosan and pentosan in the polysaccharide, the reducing power of the solution was expressed as absolute amounts of hexose and pentose sugars by reference to calibration curves, and the actual amount of polysaccharide was obtained by

multiplication of the corresponding monosaccharide values by $\frac{162}{180}$ for hexoses and $\frac{132}{150}$ for pentoses. Such calculation has given values of 95% carbohydrate for the preparations, and only in two cases, tall fescue and Brachypodium were values as low as 90% Since, according to Pirt & Whelan (1951) destruction of approximately 3% carbohydrate is to be expected by the hydrolytic method employed, it is clear that the purity of those preparations is usually approximately 98% and in no case does it fall below 93%.

Hydrolysis of the polysaccharide to its monosaccharide units was carried out by refluxing in N H_2SO_4 for three hours (1 ml. acid/mg. gum). If sufficient was available, 20 mg. portions were used for hydrolysis but determinations were satisfactory with 10 mg. portions. After cooling, the hydrolysate was neutralised with N NaOH using methyl orange or methyl red as indicator and made to a known volume. After withdrawing aliquots for determination of purity, four volumes 95% ethanol were added to precipitate Na_2SO_4, which must be removed to permit good chromatographic separation of the sugars. After standing for at least ten minutes so that the salt became thoroughly coagulated, the solution was filtered off, concentrated to a small volume and applied to chromatographic paper as already described for investigation of free sugars. Glucose, arabinose and

xylose, the sugars most frequently encountered in such hydrolysates were used as reference sugars. Both qualitative and quantitative analyses were carried out, chromatograms for qualitative analysis being prepared on No. 1 Whatman paper and irrigated with butanol/acetic acid/water solvent, while those for quantitative analysis were usually prepared on 3MM paper and irrigated in butanol/ethanol/water solvent. The problem of acquiring an absolutely reliable technique of chromatographic separation presents some difficulty and, accordingly, some practical details of the separations and of preparation of suitable solutions are included in Appendix I.

Results

The sugar units detected in gum hydrolysates and their proportions are given in Table 7 and yields of anhydro sugar units as mg. per 100 g. of dry seed in Table 8. It is seen that three main sugar residues are present, glucose, arabinose and xylose, of which glucose is by far the most abundant. Of other sugars, galactose was detected only twice and mannose once, though it should be borne in mind that this method of analysis may result in failure to detect a sugar residue present in very small proportion, which might be detected after further fractionation. A further difficulty arises from the fact that fructose, mannose and arabinose have almost indistinguishable

chromatographic mobility in butanol/acetic acid/water. Resolution of mannose from fructose and arabinose was effected by elution of the mixed spot and rerunning in phenol/water as solvent, but the complete separation of fructose and arabinose was not possible by paper chromatographic methods. The use of α-naphthol/phosphoric acid as a spray reagent occasionally indicated the presence of a small quantity of fructose in the gum hydrolysates, but in no case was the concentration sufficiently high to allow its clear detection with aniline oxalate. It is noteworthy that several preparations have unusually high "arabinose" content and this figure may be accounted for by the presence of fructose, not readily detected. It will, however, be observed that this high percentage has always occurred in very small preparations, and in the absence of a more sensitive ketose reagent, the matter has not further been investigated. In agreement with previous reports, sugars in trace, staining pink with aniline oxalate, and with R_f of approximately 0.30 and 0.34 have occasionally been detected. It is felt that the detection of trace quantities of those sugars and of fructose and mannose should be treated with reserve, particularly because of the care required, in this procedure, to prevent slight epimerisation of sugars during preparation of the hydrolysate for

Table 7

Yields, Physical characteristics, and Constituent Sugar units of Water-soluble Polysaccharides

Preparation[1]	Yield (mg per 100g.)	α_D	Specific viscosity[2]	% Sugars[3] after hydrolysis			
				G	X	A	Other
B. sterilis I	939	-71	0.71	34	41	25	-
B. sterilis II	950 (Fibrous)	0	0.60	77	9	14	-
B. mollis I	558	-32	1.96	38	45	17	-
B. mollis II	737 (Fibrous)	+1	1.53	69	10	21	-
B. asper I	625	-85	0.95	14	63	23	-
B. asper II	535 (Fibrous)	-47	0.72	43	37	20	-
Brachypodium	130	0	0.69	52	17	31	-
Agropyron	780	+85	0.17	81	9	10	-
Elymus	390 (Fibrous)	+25	0.70	56	23	21	ψ
Glyceria	190	+105	0.11	72	3	25	-
Festuca	950 (Fibrous)	+15	0.80	78	9	13	-
Lolium	850	+90	0.29	88	7	5	-
Poa	280	+31	0.18	62	9	29	-
Dactylis	1140 (Fibrous)	-10	0.65	89	2	9	-
Cynosurus	800	+50	0.27	84	7	9	-
Arrhenatherum	590 (Fibrous)	0	0.43	85	4	11	ψ
Avena	1030 (Fibrous)	+10	1.26	90	4	6	-
Holcus	180	-	-	90	4	6	-
Anthoxanthum	360	+135	0.05	88	2	10	-
Phalaris/							

Table 7 (contd.)

Preparation[/]	Yield (mg per 100g.)		Specific viscosity[//]	% Sugars[*] after hydrolysis			
				G	X	A	Other
Phalaris	170	+110	0.15	92	2	6	-
Ammophila	160	-	-	72	12	16	-
Agrostis	230	+97	0.19	78	7	15	-
Phleum	180	+70	0.13	85	7	8	-
Nardus	700	-	-	75	1	5	Mannose 19
Molinia	180	+35	0.10	50	6	21	Galactose 23
Sieglingia	110	-	-	73	6	21	-
Spartina	680	+100	0.15	93	1	6	-
Setaria	230	+132	0.02	95	1	4	-

[//] Specific Viscosity at 25°C.

[*] G - glucose; X - xylose; A - arabinose.

[/] For Bromus grasses, I is preparation precipitated by Fehling's solution, II is precipitated by subsequent addition of acetone.

[ψ] Trace quantities of galactose present.

Preparations not marked fibrous were pulverulent.

Table 8

Yields of Anhydrosugar Units

(mg. per 100 g. of dry seed)

Preparation	Glucosan	Xylan	Araban	Other
B. sterilis I	319	385	235	-
B. sterilis II	731	86	133	-
B. mollis I	212	251	95	-
B. mollis II	508	74	155	-
B. asper I	87	394	144	-
B. asper II	230	198	107	-
Brachypodium	68	22	40	-
Agropyron	632	70	78	-
Elymus	218	90	82	-
Glyceria	136	6	48	-
Festuca	741	86	123	-
Lolium	748	59	43	-
Poa	174	25	81	-
Dactylis	1015	23	102	-
Cynosurus	672	56	72	-
Arrhenatherum	501	24	65	-
Avena	927	41	62	-
Holcus	162	7	11	-
Anthoxanthum	317	7	36	-
Phalaris	157	3	10	-
Ammophila	115	19	26	-
Agrostis	179	17	34	-
Phleum/				

Table 8 (contd.)

Preparation	Glucosan	Xylan	Araban	Other
Phleum	153	13	14	-
Nardus	525	7	35	133 (Mannan)
Molinia	90	11	38	41 (Galactan)
Sieglingia	80	7	23	-
Spartina	632	7	41	-
Setaria	219	2	9	-

chromatography.

It is clear by consideration of specific rotation that in many products, e.g. that from rye-grass, much of the glucosan material is α-linked; certain other preparations, however, notably those from wild oats and Arrhenatherum, which are substantially glucosan, display a low specific rotation suggesting the presence of a β-glucosan molecule. Although arabinose and xylose were, in fact, detected in hydrolysates from every preparation, only a minority contained more than the smallest quantities of pentoses. Elymus gum consisted of approximately 50% pentosan while Agropyron gum, although containing only 20% pentosan, was obtained in greater yield and consequently had a similar pentosan content. Apart from those, each of the Bromus species examined, particularly Bromus asper, was rich in gum which has a high pentosan content. Those preparations were further distinguished by precipitating partially with Fehling's solution alone and accordingly yielding two fractions; that precipitated by Fehling's alone and designated I, had a very high pentosan content and the fraction subsequently precipitated by addition of acetone, designated II, contained a much higher proportion of glucosan. A high negative rotation was observed for the pentosan-rich fractions and it was further observed that they were much more readily

soluble in water than the acetone precipitated fraction.

Attention is drawn to other preparations which had somewhat unusual compositions. Elymus gum was found to contain a very small proportion of galactose residues while Molinia gum, which was obtained in poor yield, had a high content of both galactan and araban. With regard to the Nardus preparation, it was distinguished by containing approximately 20% mannan and a negligible quantity of pentosan; only in this preparation was mannan detected. In no case was uronic acid detected, though again it is possible that chromatographic investigation failed to detect small amounts.

Ammonium Sulphate Fractionation

Five of those preparations obtained by precipitation with Fehling's solution and acetone, including the two from Bromus sterilis, were subjected to treatment with ammonium sulphate in a manner similar to that described by Preece & Hobkirk (1953). The samples selected for treatment had all been obtained in fairly high yield and included both pentosan-rich material, and glucosan-rich materials, one of which was suspected to be mainly starchy in nature, and others in which the presence of a high quantity of β-glucosan seemed possible. The results of a sixth fractionation, which was not so completely carried out, are also quoted, although in some

respects they can not be absolutely comparable with the others. It was hoped that the composition and physical properties of fractions obtained would allow a fuller understanding of the complexity of the original polysaccharide preparations.

Method

Quantities varying from 0.75 g. - 1.2 g. were used for fractionation. The gum was dissolved in water at about 80°C. with stirring to give a solution of approximately 1% concentration. The solution was then cooled to and kept at 15°C. while 20% ammonium sulphate (w/v) was gradually added and dissolved. Ample time was allowed for precipitation to occur and, particularly if the solution was turbid, it was left for as long as several hours. Any precipitate was removed by centrifuging and more salt, 10% at a time, was added until the solution was saturated with respect to ammonium sulphate. The mother liquor was reserved for dialysis. After each addition of salt, the solution was centrifuged and the precipitate removed. Those precipitates were redissolved in a small volume of hot water and reprecipitated by addition of acetone to 60% concentration. This step was intended to remove colouring matter from the carbohydrate in addition to a certain amount of ammonium sulphate. Each precipitate was again dissolved in water and increasing quantities of

ammonium sulphate were added to each of the solutions. Precipitates at the same salt concentration were combined and after redissolving were once more treated with acetone. This process was repeated once more so that a total of three precipitations with salt was carried out. The restricted fractionation, already referred to, was of rye-grass gum which was subjected to only one treatment with ammonium sulphate. If, at any stage, a precipitate was very small it was not subjected to further treatment lest its recovery might prove impossible. Finally, the fractions were dissolved in water and dialysed against running water, in presence of thymol. The solutions were dialysed for three days after which a small portion was tested for sulphate by means of barium chloride. The mother liquor was also dialysed, in this case for four or five days. After dialysis, the solutions were filtered through a pad of kieselguhr and the carbohydrate was precipitated by addition of acetone. Not more than two volumes of acetone were used though it was, on occasion, difficult to effect coagulation of the smaller precipitates. This was assisted by shaking and by placing in the refrigerator for a period. Mother liquor solutions were first concentrated to a small volume before addition of acetone and, with those, as much as five volumes of acetone was used if required. The precipitates were

then all washed in aqueous acetone followed by alcohol and transferred to a sintered glass crucible. After drying overnight in vacuo and thereafter at 100°C. the preparations were weighed and transferred to stoppered tubes.

Properties and composition of fractions

Fractions obtained were usually white in colour and determination of carbohydrate content showed that this normally approached 100%. Exceptions were the tall fescue fractions which all retained slight colour and all mother liquor fractions which were either cream or slightly brown in colour. Those fractions were always extremely small in size and obviously contained much of the small quantity of impurity present in the original mixture. The carbohydrate content of the tall fescue mother liquor fraction was as low as 60%. Recoveries of those fractions, corrected for impurity, are reported in Table 9. In appearance, the fractions were either fibrous or pulverulent; in general those fractions obtained at

Note: For the sake of convenience and clarity, fractions precipitated by 20% salt concentration will be referred to as "20% fractions" and similarly for those up to "70% fractions". The fraction recovered from the mother liquor will be called "the mother liquor fraction".

ammonium sulphate concentrations of 20%, 30% and to a lesser degree at 40%, were fibrous, while the fractions obtained by addition of more salt were powdery. Notable exceptions were the cocksfoot 20% and 30% fractions both of which were tightly packed so that they could not satisfactorily be ground, though no particular significance, at the moment, can be attached to this observation. In every case, those fractions precipitated at high salt concentration were the most soluble in water; glucosan-rich fractions precipitating at 20% or 30% concentration do not possess high water-solubility.

Colouration with iodine varied very much, certain of the fractions still developing a blue colour. Iodine colouration with 20% fractions was either blue-green or faintly green, while fractions obtained at high salt concentration usually produced a brown or reddish-brown colour. Table 9 contains a list of the colouration of fractions with iodine.

Specific rotation and viscosity readings were not always obtainable on account of the small yields of some of the fractions but where possible those determinations were carried out and are reported in Table 9. Viscosity measurements show a distinct tendency to decrease from fractions obtained at low salt concentration to those obtained at high concentration. The measurement of specific rotation

proved very much easier for those fractions than for the preparations obtained with Fehling's solution and acetone, although some difficulty was still encountered with 20% fractions, allowing an accuracy of not better than $\pm 2^{\circ}$. Specific rotations of pentosan-rich fractions were particularly accurate.

Hydrolysis of fractions was carried out with H_2SO_4 as already described and the relative amounts of anhydro-sugar residues determined by chromatographic separation and estimation with the copper-reducing reagent. Those values are quoted in Table 9.

Results

It will be seen that absolutely pure glucosan has been obtained in the 20% fractions with the exception of the small fraction obtained from tall fescue gum which contained 6% pentosan. Such small pentosan contamination is probably of little significance, though it seems a little surprising that the fractionation technique did not remove all this contamination, in view of the fact that pentosan content of the original gum is not high. Possibly further precipitation would have removed it. For those fractions, specific rotations do not vary greatly from the figure of -13° quoted for barley β -glucosan. Of the grass seeds here examined, wild oats is a very abundant source of this gum fraction. It will be observed that the fractions obtained at 30% concentrations again have

1953). Of the mother liquor fractions, which are distinguished by their smallness, none has been observed to contain galactan, while araban content is only slightly higher than in other fractions.

Restricted Ammonium Sulphate Fractionation

It was thought desirable to subject a greater number of the gums to treatment with ammonium sulphate. Previous fractionations appear to have established the potentialities of this treatment with regard to fractionation of the gums, and, therefore, a severely modified procedure was considered sufficient to show any marked trend in fractionation, in particular to detect β-glucosan the bulk of which was believed to precipitate with a 30% concentration of salt.

Five gums were subjected to the following modified procedure: to a 1% aqueous solution of the gum at 15°C. was added ammonium sulphate to a concentration of 30% and any precipitate removed. The salt concentration was then raised to 50% and any further precipitate removed and the mother liquor dialysed against running water for four days. The precipitates obtained were dissolved in water, precipitated with two volumes of acetone, redissolved and dialysed for three days. All fractions were then recovered and taken to dryness exactly in the way previously described.

20 mg. portions were hydrolysed in N H_2SO_4 and

the relative quantities of sugar units determined. Recoveries and compositions of the fractions are reported in Table 10. It is observed that three fractions are obtained by this procedure and to avoid confusion with fractions obtained by the full procedure, they are referred to as fractions "a", "b" and "c". Fraction "a" corresponds to a mixture of 20% and 30% fractions, "b" to 40% and 50% fractions and "c" consists of 60%, 70% and mother liquor fractions of the full procedure.

Results

Three of the five gums yielded "a" fractions, though that from crested dog'stail gum was very small. The specific rotation of the "a" fraction of Bromus mollis gum was -6° and of Arrhenatherum gum was -9°. Each of those fractions had a very high glucosan content and those observations indicate that the glucosan is substantially β -linked. Marked fractionation of the Bromus mollis gum was achieved, fractions "b" and "c" being predominantly pentosan. The failure to obtain "a" fractions from Elymus and Agropyron gums points to the absence of any appreciable amount of β -glucosan. Galactan which was detected in trace in Elymus gum was more readily detected after fractionation. Arrhenatherum gum, in which galactan had not been detected, yielded a "c" fraction containing relatively high quantities of

Table 9

Composition and Properties of the Fractions obtained using Ammonium Sulphate

Fraction precipitated by $(NH_4)_2SO_4$ concentration shown	Recovery %	α_D	Specific viscosity ≠	Anhydrosugar units %			Colour with Iodine
				Glucosan	Xylan	Araban	
B. sterilis I							
30%	13	-11	0.59	94	2	4	Blue
40%	17	-96	0.39	10	64	26	Blue
50%	12	-102	0.46	9	65	26	Red-Brown
70%	5	-101	0.38	12	59	29	Red-Brown
M.L.	3	-48	-	15	65	20	Brown
B. sterilis II							
20%	30	-8	0.80	100	0	0	Faint Green
30%	4	-9	0.55	99	0	1*	Blue
40%	4	-17	0.51	58	27	15	Red-Blue
60%	1	-	-	81	13	6	Red-Blue
M.L.	1	-	-	60	25	15	-
Festuca							
20%	9	-4	2.03	94	4	2	Blue
30%	15	-12	1.05	85	10	5*	Blue
40%	3	+36	-	55	28	17	Blue
50%	4	+50	-	75	16	9	Brown-Blue
70%	1	-	-	80	13	7	Red-Brown
M.L.	9	-	-	75	5	20*	Red-Brown
Lolium							
30%	9	-	-	82	12	6	Blue
40%	31	+103	0.30	85	9	6	Red-Blue
50%	8	-	-	89	6	5	Red
70%	9	-	-	88	4	8	Red
M.L.	5	-	-	83	10	7	Red
Dactylis							
20%	19	-9	1.10	100	0	0	Blue
30%	18	-7	0.59	99	0	1*	Green-Blue
M.L.	7	+25	0.05	64	12	24	Red-Brown
Avena							
20%	29	-8	1.95	100	0	0	Blue-Green
30%	12	-8	0.85	100	0	0	Green-Blue
40%	4	0	0.60	94	4	2	Blue
50%	3	+18	-	80	12	8	Green-Blue
70%	2	+71	-	70	14	16	Red-Blue
M.L.	3	+88	0.03	86	2	12*	Red-Brown

* Includes Hexosan

≠ 0.5% solution, 25°C. (in water)

Table 10

Composition of the Fractions obtained using the Restricted Ammonium Sulphate Method

Fraction	Recovery %	Anhydrosugar units %				Colour with Iodine
		Galactan	Glucosan	Xylan	Araban	
B. mollis*						
a	28	-	84	11	5	Green-Blue
b	11	-	46	35	19	Blue
c	5	-	32	49	19	Red
Agropyron						
a	0	-	-	-	-	-
b	28	-	86	7	7	Red-Blue
c	15	-	68	6	26	Red
Elymus						
a	0	-	-	-	-	-
b	37	-	55	30	15	Blue
c	8	17	33	20	30	Red
Cynosurus						
a	5	-	80	11	9	Blue
b	19	-	80	11	9	Blue
c	27	-	80	8	12	Red
Arrhenatherum						
a	38	-	89	3	8	Green-Blue
b	15	-	86	6	8	Blue
c	10	22	39	11	28	Red

Specific rotations Bromus mollis "a" -6°

Arrenatherum "a" -9°

* B. mollis preparation is a mixture of B. mollis I and B. mollis II with composition

Glucosan	56%
Xylan	25%
Araban	19%

Table 11*

Recoveries of Polysaccharide after Ammonium Sulphate Fractionation

Preparation	Total Recovery %	Recovery of anhydrosugar units %		
		Glucosan	Xylan	Araban
B. sterilis I	50	46	59	40
B. sterilis II	40	49	17	6
Festuca	41	43	45	29
Lolium	62	60	80	74
Dactylis	44	47	43	21
Avena	53	57	21	17
B. mollis	44	54	38	23
Agropyron	43	42	32	59
Elymus	45	41	55	38
Cynosurus	51	49	70	59
Arrhenatherum	63	60	77	65

* Results calculated by reference to tables 7, 9 and 10.

Table 12

Ratio of Xylan to Araban in Pentosan-rich Fractions

Fraction		Ratio x : a
B. sterilis	40%	2.5 : 1
	50%	2.5 : 1
	70%	2.0 : 1
B. mollis	b	1.8 : 1
	c	2.6 : 1
Elymus	b	2.0 : 1

galactan and araban, and in this way, resembling the mother liquor fractions of the cereal gums.

Hemicelluloses

Investigation has already been carried out (Preece & Hobkirk, 1954) to observe relationships between the water-soluble gums and soda-soluble hemicelluloses of some of the cereals. The hemicelluloses, soluble in 4% soda, have now been extracted from four samples of grass seeds with a view to comparing them to the gums already extracted.

The grass seeds selected for this investigation were canary seed, which was virtually gum-free, wild oats having a high β-glucosan gum content, Elymus having a high pentosan gum content, and finally Bromus mollis which had a high content of both glucosan and pentosan gums.

Experimental

The ground seeds, which had been refluxed in alcohol and extracted in water, were subjected to a process of autoclaving to gelatinise the starch present followed by treatment with malt α-amylase to degrade the starch. The α-amylase was prepared by the method of Preece & Shadaksharaswamy (1949) in the following way: the ground malt was extracted in 20% ethanol and the enzyme precipitated by adjusting the alcohol concentration to 60%. The precipitate was dissolved in water, calcium acetate (0.2 g./100 ml.)

added, and the solution heated to 70°C. and maintained at that temperature for fifteen minutes. After filtration, the enzyme was reprecipitated and taken to dryness. This treatment is believed to inactivate all the enzymes present with the exception of α-amylase. The preparation has been shown to have no action on β-glucosan or water-soluble arabo-xylan.

The extracted seeds were suspended in water (100 g. in 400 ml.) which was brought to boiling point with stirring. The suspension was then subjected to a pressure of 15 lb./sq.in. at 120°C. in an autoclave for thirty minutes, and then cooled to 65°C. A small quantity (about 20 mg.) α-amylase, dissolved in water was then added. The solution was maintained at 65°C. for three to four hours to allow breakdown of the gelatinised starch and thereafter the autoclave and enzyme treatments were repeated several times until no starch could be detected. Absence of starch was judged by failure of the solution or fragments of the seed to produce colouration with iodine. The destarched residue was washed with boiling water to remove dextrins and sugars and the water removed either at the pump or by centrifugation. The seeds were then extracted with 4% sodium hydroxide at 10°C. Three thirty-minute extractions (500 ml., 250 ml., and 250 ml./100 g. seed)

were carried out with constant stirring. The combined solutions were filtered as quickly as possible through paper pulp until the liquid was water-bright. One-half volume of Fehling's solution was added to the soda extract, and any precipitate removed. Acetone, to 40% of the volume of the solution was then added and the precipitate again removed. The precipitates, separately, were dissolved as far as possible in N H Cl and after reprecipitation with acetone were washed and taken to dryness as for the gums.

After hydrolysis of 20 mg. portions in N H_2SO_4 for three hours (this treatment was sufficiently powerful to effect complete hydrolysis to monosaccharide units) the sugar residues were detected chromatographically and their relative amounts determined.

Results

Yield and composition of the preparations are given in Table 13. The preparations which were white or greyish-white in colour, all dissolved slowly in hot water. It will be observed that yields are approximately three times as great as the corresponding gum yields. With the exception of the hemicelluloses of canary seed, all partially precipitated without the addition of acetone, and all had a high pentosan content. Wild oats hemicellulose, particularly was

Table 13

Yields and Compositions of Hemicelluloses

Preparation*	Yield % dry wt. of seed	Anhydrosugar units %			Ratio x : a
		Glucosan	Xylan	Araban	
B. mollis I	4.08	11	71	18	4.0 : 1
B. mollis II	2.67	69	24	7	3.4 : 1
Elymus I	3.56	7	68	25	2.7 : 1
Elymus II	1.22	41	37	22	1.7 : 1
Avena I	4.80	8	78	14	5.5 : 1
Avena II	0.26	48	39	13	3.0 : 1
Phalaris	1.59	34	43	23	1.9 : 1

* Preparation I : Fehling's Precipitated
Preparation II: Precipitated by subsequent addition of acetone

Table 14

Yields of Anhydrosugar units of Hemicelluloses

(mg. per 100 g. of dry seed)

Preparation	Glucosan	Xylan	Araban
B. mollis I	450	2900	730
B. mollis II	1840	640	190
Elymus I	250	2420	890
Elymus II	500	450	270
Avena I	380	3750	670
Avena II	130	100	30
Phalaris	540	680	370

almost exclusively pentosan in nature, but glucosan was detected in each preparation. It is clear that some of this was starchy, since faint blue colouration was obtained with each preparation, but nevertheless the majority of it is believed to be hemicellulosic.

A spot, with a very low mobility in butanol/ethanol/water and with an R_f value of about 0.14 in butanol/acetic acid/water, has been detected on chromatograms of hemicellulose hydrolysates. The presence of this spot which was distinct only on chromatograms of the hydrolysate of wild oats hemicellulose points to the presence of galacturonic acid. In view of the fact that the amount of uronic acid involved was clearly very small, no attempt was made to estimate its concentration.

Aqueous Extraction of Non-starchy Polysaccharides in an Autoclave

Experimental data has been obtained concerning those polysaccharides extracted by water at 40°C. and details are also given, for four cases, of the hemicelluloses thereafter extracted by sodium hydroxide. Evidence is accumulating in favour of the view that certain of the hemicellulosic material extracted by soda is very closely related to the gums obtained by aqueous extraction at 40°C., this temperature being used because it conveniently allows the extraction of gums without having the effect of

gelatinising the starch grains of the seed. Any polysaccharide with solubility intermediate between the gums and the hemicelluloses would be brought into solution along with the starch during autoclave treatment. To investigate any non-starchy carbohydrate thereby extracted, it is first necessary to remove the starch, this being accomplished by use of an α-amylase preparation, which does not attack hemicellulosic polysaccharides. The products of starch degradation require to be removed by dialysis before an attempt is made to recover any remaining polysaccharide.

Attempts were made to isolate non-starchy polysaccharides at this stage from extracts of the four grasses from which sodium hydroxide extractions were made.

Procedure

After alcoholic and aqueous extraction, the residue was destarched by successive treatments in the autoclave followed by enzymic degradation of the gelatinised starch. The details of this treatment are reported in the preceding section on hemicelluloses. The total amount of α-amylase used was kept as low as possible, and for a 50 g. sample of seed approximately 25 mg. were required. The combined solutions containing degraded starch were kept for four days at 52°C., in the presence of thymol to

obtain as complete degradation as possible. The solution was then dialysed against running water, again with thymol present. After dialysis, the solution was filtered, concentrated on a water-bath (to 250 ml. for an extract of 50 g. seed). The carbohydrates were then precipitated by addition of Fehling's solution and acetone and then taken to dryness.

Extracts were made of Bromus mollis, Elymus, wild oats and canary seed. After dialysis the solution obtained from B. mollis was highly viscous while the other three appeared to have a low viscosity. On concentration of the Bromus extract and to a very much lesser extent of the Elymus solution, skin formation occurred. A large gummy precipitate was obtained from the B. mollis solution after addition of both Fehling's solution and acetone. A very much smaller precipitate was obtained from the Elymus extract but no workable yield was obtained from either wild oats or canary seed solutions.

The two preparations were fibrous in appearance and white in colour and each redissolved in water at about 80°C. Neither gave any colouration with iodine. Determination of the carbohydrate content by the Somogyi method showed that they both contain over 90% carbohydrate, a slightly lower purity than that obtained for the gums. The B. mollis preparation

which had a specific rotation of -40° (uncorrected for impurity) had a high glucosan content, while the Elymus preparation consisted largely of pentosan. No galactose or uronic acid was detected on hydrolysis.

It was determined that the α-amylase used had approximately a 50% carbohydrate content which consisted of 10% glucosan and equal amounts of araban and xylan. It was therefore possible for an extract from 50 g. seed that a maximum of 13 mg. of the enzyme preparation might be recovered as carbohydrate. This would amount to 6% of the Elymus preparation and to a negligible proportion of the Bromus preparation.

A Bromus extract was subjected to ammonium sulphate treatment, only one addition of salt being made. A very large fraction was obtained at 20% concentration, consisting predominantly of glucosan and having a specific rotation of -14°. A 30% fraction and other very small fractions, rich in pentosan were thereafter obtained. Details of products obtained are given in Table 15.

Results

The preparation obtained from Elymus seeds by this extractional method was substantially pentosan and in this way resembled both the gum and the hemicellulose.

The preparation from B. mollis had a much higher glucosan content and again appeared to be very similar

Table 15

Yields and compositions of preparations

(Autoclave extracted)

Preparation	Yield % dry seed	α_D	Anhydrosugar units %		
			Glucosan	Xylan	Araban
*Elymus	0.534	-	23	54	23
B. mollis	7.260	-40	72	19	9
✓B. mollis 20%	3.262	-14	86	6	8
B. mollis 30%	0.862	-	22	55	23
B. mollis 50-70%	0.176	-	40	45	15
B. mollis M.L.	Trace	-	-	-	-

*Precipitated by use of Fehling's Solution and Acetone

✓Precipitated by use of $(NH_4)_2SO_4$

to the corresponding water-soluble gum. Its physical characters, particularly specific rotation, and behaviour with respect to $(NH_4)_2SO_4$ show that a very large part of the glucosan must be β -linked, while the fact that no product was obtained from two other grasses is further evidence that the amylolytic treatment used satisfactorily removes the starch content.

These findings indicate the extremely high content of hemicellulose-type carbohydrate in the seeds of B. mollis, this material extracted by water and 4% NaOH amounting to approximately 14% of the dry weight of the seed. The presence of such carbohydrates with solubility intermediate between the gums and the hemicelluloses, but clearly with very marked similarity to each, is additional evidence of the close relationship between the gum and hemicellulosic materials.

DISCUSSION

Preparations precipitated by use of Fehling's solution and acetone

In an assessment of the nature of those preparations, attention is first directed towards their simple physical characteristics and to their yield. With regard to yield, preparations have been as small as 0.1% and as great as 1.9% of the dry weight of the seed, although for reasons already enumerated with regard to sugar content, it is impossible to consider those yields absolutely comparable. It has been shown that the content of water-soluble polysaccharides in the husks of barley and wheat is very small, and aqueous extraction of small quantities of the husk of wild oats and Elymus seeds has failed to yield any carbohydrate. There seems ample evidence, therefore, to regard water-soluble carbohydrates as being absent, or virtually so from the seed husk. Although absolute comparison is impossible, reference to Tables 1 and 7 shows that yields appear to bear no relationship to the volume of the seeds. Thus, although a high yield is obtained from wild oats seeds, a very small yield is obtained from canary seeds, which are fairly large. On the other hand, cocksfoot seeds have a high gum content and are very small in size.

Consideration of the physical properties shows

very marked differences. Preparations may be either fibrous or powdery in nature. Fibrous preparations in general are distinguished by the fact that they are more viscous, have a negative specific rotation and tend to form a skin on being concentrated in aqueous solution. Other non-fibrous carbohydrates, on the other hand, have no marked viscosity and have a high positive rotation. Fibrous preparations are found to consist of either glucosan or pentosan carbohydrate while the pulverulent type always consist substantially of glucosan. It is clear that those findings suggest that the polysaccharide entities present in those materials have compositions and properties similar to those encountered in a similar investigation on cereal gums. There is evidence of the presence of glucosan, dextrorotatory and resembling starch, along with glucosan and pentosan polysaccharides with properties resembling those of the cereal gums. The different proportions of those molecular entities are so marked as to be noticeable even without fractionation of the mixtures.

Ammonium sulphate fractionation

The results now obtained by application of this technique are further evidence of the fractionation which may be achieved by its use. Observations previously made, that a mixture of β -glucosan and pentosan may be separated by this means, are upheld.

It is clear that β-glucosan is substantially precipitated by a minimum concentration of 20% ammonium sulphate and by a maximum of 30% salt. It is also observed that the water-soluble pentosans of grass seeds precipitate at concentrations ranging from 40% to 70% salt. While such separations do effectively occur, there is a tendency for a restricted amount of mixing of those two molecular species, with the result that even 20% fractions after one salt treatment frequently contain a small amount of pentosan. Further precipitations are invariably capable of separating the pure glucosan but there is much less certainty of obtaining an absolutely pure pentosan. It seems clear that the bulk of contamination in pentosan-rich fractions is α-glucosan in nature and evidence in favour of this point of view is obtained by treatment of such fractions with α-amylase. Those preparations, for example the pentosan-rich fractions of Bromus sterilis, invariably stain blue with iodine, but on treatment with malt α-amylase, the preparations, on reprecipitation, give no colour with iodine. Furthermore, oligosaccharides of the maltose type, were detected chromatographically in the solutions after such enzyme treatment. In spite of amylase treatment, however, it is still found difficult to remove the glucosan content completely. The complete

absence of colour on addition of iodine to such a preparation, which had been subjected to prolonged dialysis, suggests that the final glucosan remnant is a β-linked molecule.

That β-glucosans are substantially precipitated at a maximum of 30% salt concentration is shown by consideration of the compositions and specific rotations of those fractions, and further by the fact that a glucosan-rich fraction obtained from rye-grass by 40% salt has a specific rotation of $+103^{\circ}$ and is therefore quite different in type. It should be emphasised that the striking fractionation between fractions precipitated below and above a salt concentration of 30% is satisfactorily obtained on treatment of carbohydrate extracted in water at 40°C. Similar treatment of hemicellulosic material, rendered water-soluble by the extractional treatment, resulted in a similar but rather less clear-cut fractionation (Preece & Hobkirk, 1954). The outstanding point of difference was the fact that pentosan material tended to precipitate at a lower salt concentration so that repeated precipitations failed to give a pure glucosan even at the 20% level. Furthermore, 30% fractions usually had a high pentosan content. A similar observation is now obtained on fractionation of the gum-like material obtained by aqueous extraction in an autoclave of Bromus mollis seeds. In this

instance a marked fractionation between glucosan and pentosan was obtained by separation of the 20% and 30% fractions.

The results of the present studies differ from those of ammonium sulphate fractionation of the cereal gums most noticeably in the virtual absence of galactan material. Small quantities of galactan were present in mother liquor fractions of each of the cereal extracts, whereas in only three instances has galactan been detected in the grass seed preparations. There seems no doubt that the absence of galactan in most cases is explained by failure to recover it during the Fehling's treatment. This is consistent with the fact that mother liquor fractions were invariably very small, and with the observation (Hobkirk, 1955) that approximately 80% of water-soluble carbohydrates are recoverable by use of Fehling's solution and acetone. It is concluded that the preparative method employed results in recovery of all but that material corresponding to the bulk of ammonium sulphate mother liquor fractions, which normally consist of dextrinous and pectic substances.

Recoveries. Gum preparations from the common cereals have been precipitated both by the use of Fehling's solution and acetone (Preece & Mackenzie, 1952b) and by ammonium sulphate (Preece & Hobkirk, 1953), and although comparable results are not accurately

available, it is clear that precipitation using ammonium sulphate results in yields of approximately 50% of those obtained by precipitation using Fehling's solution. The procedure now adopted, whereby preparations first obtained by the latter process were redissolved and subjected to salt fractionation, allows a more reliable assessment of recoveries to be made. Reference to Table 11 shows that in fact recoveries vary from as little as 41% to a maximum of 63%. Most treatments, however, resulted in a recovery of little more than 50% and it is noticeable that little difference exists in the recoveries obtained after only one salt treatment and after three treatments respectively. It seems, therefore, that although successive precipitations would in all likelihood involve some small losses as a result of the carbohydrates retaining a slight solubility in the precipitating solution, the major loss of material must occur during dialysis of the fractions.

Mechanism of precipitation. Some brief reference might be made to the mechanism involved in the precipitation of carbohydrates by ammonium sulphate. Although it has been of standard use for the precipitation of proteins, only recently has ammonium sulphate been used in carbohydrate fractionations. It may be that the salt has the effect of dehydrating the carbohydrate in solution and thereby causing its

precipitation, but in fact there is no knowledge of the precise factors involved. It is clear, however, that precipitation is influenced profoundly by the water-solubility of the macro-molecule, since for example β-glucosan is much less soluble in water than the pentosans. The concentration of the carbohydrates in solution however appears to have very little effect. It is most striking that β-glucosan preparations of vastly differing viscosities are all precipitated by 25% or little more ammonium sulphate. Preece & Hobkirk (1953) did show that, for a β-glucosan mixture, material of lower viscosity was precipitated as the salt concentration was increased from 20% to 30%. This observation, however, is made over a restricted range and does not detract from the rather surprising fact that β-glucosan preparations, some with specific viscosities as high as 10, others as low as 0.5 all precipitate at substantially the same salt concentration. It is certain that increasing viscosity represents an increasing molecular size and wide difference of this nature might have been expected to cause distinct variation in levels of precipitation.

Viscosity of preparations

The viscosities of the water-soluble preparations varied very considerably but over a somewhat restricted range. Those preparations of high

positive specific rotation, and containing polysaccharides of a starchy nature, have virtually no viscosity. On the other hand, a specific viscosity of 1.96 is observed for a Bromus preparation. On measurement of the viscosities of ammonium sulphate fractions it becomes clear that the viscosity of the mixed preparation is an average value of the constituent molecular types present. In general, the 20% fraction is the most highly viscous and consequently this fraction is always more viscous than the mixture from which it was separated. The viscosity of fractions usually falls off at higher salt concentrations, although pentosan-rich fractions, obtained at 40% and 50% concentrations, may also be highly viscous. Mother liquor fractions invariably have virtually no viscosity.

Although the viscosity differences are quite distinct, it is necessary to consider why in fact comparatively low viscosities are obtained throughout. As stated earlier, specific viscosities of over 10 are frequently reported for barley β-glucosan, and even more highly viscous preparations of rye pentosan have been obtained. The viscosities of such purified fractions can only legitimately be compared to ammonium sulphate fractions, and of those wild oats and tall fescue 20% fractions have specific viscosities of 1.96 and 2.04 respectively. In earlier

preparations of cereal gum fractions, rather low viscosities, only infrequently exceeding these values, were observed. Two reasons are suggested for the failure to obtain a highly viscous preparation after precipitation with ammonium sulphate. First, differences will almost certainly exist in the state of the polysaccharide in the grain, and it may very well be that this factor has a strong influence on viscosity. Equally important, however, is the necessity to effect complete inactivation of enzymes before aqueous extraction is commenced, because cytoclastic activity remaining in the solution would rapidly reduce its viscosity.

In the present investigation, enzyme inactivation has been shown to be adequate to allow the preparation of material of high viscosity so that this factor may safely be ignored. It seems not unlikely that the molecular aggregation and therefore the resultant viscosities of the polysaccharide will differ throughout the range of wild grasses investigated. It is clear however that precipitation of the carbohydrates by means of the Fehling's technique is responsible for the most drastic reduction in viscosity.

Effect of Fehling's treatment on gum viscosities. There is no doubt that this treatment results in reduced viscosity: for example Bromus mollis seeds,

after enzyme inactivation, were extracted with water and the preparation obtained by precipitation using Fehling's solution with acetone and that obtained by addition of alcohol had respectively, specific viscosities of 0.82 and 3.54. It is known that alkaline solution may degrade carbohydrate, so that some viscosity diminution for this reason would be not unexpected. Nevertheless, there is some reason to believe that the effect of the alkali is not so drastic as the effect of the ensuing hydrochloric acid treatment. First, on dissolving the gum in acid, the solution is frequently highly viscous and yet after recovery the viscosity seems to have been considerably reduced. Second, very highly viscous preparations have been obtained (Preece & Hobkirk, 1954) by extraction with alkali followed by neutralisation and precipitation with acetone. It seems, therefore, that the reduction in viscosity, which undoubtedly occurs, is caused not simply by the effect of alkaline conditions but by dissolution in acid or possibly by a combination of the acid and Fehling's treatment.

It might be observed, more generally, that there is an urgent need for precise description of methods used in preparations of materials. For example, the low viscosities discussed here, appear to be the result of the preparative method employed. Only if details of methods are reported, is true comparison of

similar work possible.

Precipitation and fractionation by Fehling's solution

It is frequently found that hemicelluloses are precipitated by addition of Fehling's solution alone, this precipitation apparently involving the formation of a complex insoluble in the alkaline conditions. The present investigation shows that part of the soda-soluble material is usually precipitated in this way. Previous work had failed to obtain such precipitation of water-extracted gums of the cereals, further addition of acetone always being required. This state of affairs is found to hold throughout the present work, with the notable exception of the extracts of Bromus seeds. In each of these instances, a precipitate with Fehling's solution was obtained along with a further precipitate when acetone was added.

Some attempt has been made to determine the reason for differences in precipitation on addition of Fehling's reagent. It was observed that whether the carbohydrate had been extracted by alkali or by water, the precipitate obtained by Fehling's solution was bulky, greenish in colour and jelly-like. On the subsequent addition of acetone, a certain amount of jelly-like material was usually precipitated, but the bulk of the precipitate was white and stringy in nature. It has frequently been stated that a carbohydrate-copper

complex is obtained by addition of Fehling's solution with or without acetone, but from the following observations it is clear that two distinct types of precipitate may be obtained.

(a) Samples of pure β-glucosan (20% ammonium sulphate fractions from wild oats) were dissolved in small volumesof water to give solutions of concentrations 0.5%, 1% and 2%. More concentrated solutions were not obtainable. On addition of an equal volume of Fehling's solution, no precipitate was obtained, nor was any increase in the viscosity of the solution noticed. When acetone was added, a white precipitate was obtained.

(b) Aqueous solutions of soluble starch up to a concentration of approximately 2% were prepared and treated similarly with Fehling's solution. Again no precipitation occurred until acetone was added, when the starch was recovered as a white flocculent precipitate.

(c) A pentosan-rich fraction, containing less than 10% glucosan contamination (40% ammonium sulphate fraction from an aqueous extract of Bromus sterilis) was dissolved in similar concentrations. On addition of Fehling's solution, no precipitate was obtained from the 0.5% solution, a very slight precipitate from the 1% solution, and a heavy precipitate, green and jelly-like, was obtained from the 2% solution.

On subsequent addition of acetone to all three solutions, further precipitation of this jelly-like material occurred.

(d) To a solution containing 1% β-glucosan and 1% arabo-xylan, an equal volume of Fehling's solution was added. Again a typical Fehling's precipitate was obtained. On adding a small amount of acetone more of this material precipitated, but on further addition of acetone, the white stringy precipitate was obtained.

From those observations it is clear that two types of precipitate are obtained. There seems no doubt that the precipitate obtained by addition of Fehling's solution consists substantially of pentosan in the form of a copper complex, some glucosan contamination usually being present. Addition of acetone precipitates the remainder of this pentosan material, still as a complex. On the other hand, there is no evidence from those observations to suggest that β-glucosan or starch form a complex with Fehling's solution, although it has been reported (Rakovski, 1914) that cuprammonium solutions form copper complexes with starch.

As a result of the tendency for a pentosan-copper complex to precipitate, it has been possible to effect fractionation by use of Fehling's solution. Reference to Table 7 (for water-soluble preparations of Bromus species) and Table 13 shows that very marked

fractionation occurs between pentosan and glucosan. Furthermore, the fraction precipitated by Fehling's solution alone, always has a higher xylan/araban ratio than the other fraction. Complete fractionation by this method, however, is not practicable because of the high losses incurred by repeated treatments with Fehling's solution.

General

The extractional and fractionation processes which have been undertaken demonstrate that the water-soluble products obtained from the grasses are mixtures of several molecular types. There is sufficient evidence available to show that the material encountered in the cereal gums (Preece & Hobkirk, 1953) closely resemble those now detected in this wider survey. Although certain minor constituents, galactan, mannan, araban and fructosan, are detected, no details are available as to their chemical nature. There are three principal constituents of the water-soluble polysaccharides encountered, namely β-glucosan, α-glucosan, and arabo-xylan, and the ratio of those differs very widely in preparations from seeds of different grasses. From the somewhat restricted data available, mainly for barley, it appears, however, that the composition of the preparation is substantially constant for any one species. The results obtained for three species of

Bromus in this investigation show that while some quantitative difference exists in their yield, there is a fair measure of similarity in composition. As is the case with the sugars of the grasses, there is therefore some reason for believing that the water-soluble polysaccharides of the seeds of any species - or possibly even genus - of grass are substantially constant, although again some differences would presumably result on account of environmental difference and on the precise time of harvesting.

β-glucosan. β-glucosan in quantity has been isolated pure or almost pure from five grass species and in small amounts from two others. Of the preparations subjected to fractionation, Agropyron and Elymus failed to yield any β-glucosan. The fractionation technique undoubtedly fails to recover small quantities of β-glucosan, but recoveries should approximately reflect the amount present in the mixture. It is clear, from specific rotations, that of those grasses not subjected to fractionation none contained substantial quantities of β-glucosan, with the exception of Bromus asper and possibly meadow grass, although small amounts may possibly be present.

Specific rotations of the β-glucosans obtained are consistently slightly more positive than the value for barley β-glucosan, and are nearer the value quoted for the preparation from oats. Those slight

differences may be accounted for by the presence of small quantities of starchy material, or, quite possibly, by small differences in the ratio of 1,3 to 1,4 glycosidic links in the molecule (barley β-glucosan specific rotation -13°, lichenin from Icelandic moss, specific rotation +8°).

<u>α-glucosan</u>. Glucosan material staining blue with iodine occurs in all preparations, again in widely varying amounts. Physical appearance, viscosity and specific rotation are sufficient to show that many of the preparations consist very largely of this material.

Four such preparations (from maize, <u>Molinia</u>, <u>Setaria</u> and <u>Anthoxanthum</u>) were dissolved in water and treated with malt α-amylase. The early course of the reaction was followed by noting the colour produced by the solutions with iodine. After leaving the solutions overnight, the enzyme action was stopped. Four volumes 95% ethanol were added to the solution to precipitate high molecular carbohydrate. The solutions were then concentrated to a small volume and spotted on chromatograms. Qualitatively, the sugars produced appeared to be the same; furthermore they were the same as those produced by a control solution of starch similarly treated. Maltose was the principal sugar detected, along with maltotriose and higher oligosaccharides. Small quantities of glucose were also detected.

The close similarity of this glucosan to starch is obvious but normally starch would not be extracted by water at such a low temperature. It may be that this material resembles phytoglycogen recently obtained from maize (Peat et al., 1956). Phytoglycogen was found to have a maximum chain length of 13 units in its repeating unit and in this respect to be more similar to glycogen than to amylopectins normally found in plants.

Pentosan. Consideration of the results in tables 7 and 10 indicates that a restricted amount of free araban must be present in at least some seeds, and also that the presence of galacto-araban is possible in several instances.

Nevertheless, it is certain that the bulk of pentosan exists as an arabo-xylan. Pentosan occurs in all preparations but only to any extent in the Bromus preparations and in those from Elymus, Agropyron and tall fescue.

Although the xylan/araban ratio of pentosans obtained from the cereals was variable, it most frequently was approximately 1.7:1, (Preece & Hobkirk, 1953). The somewhat higher ratio of xylose to arabinose obtained in the pentosans of Bromus and Elymus may truly represent the molecule present in the plant, but it is more likely that the effect of using Fehling's solution in their preparation is to remove a small

proportion of the arabinose residues. Hobkirk (1955) has shown that repeated treatment with Fehling's solution may remove those residues completely from the arabo-xylan molecule.

As to specific rotation, ammonium sulphate fractions from Bromus sterilis containing approximately 10% glucosan, have been obtained with specific rotation of -100°. After treatment of the 50% fraction with α-amylase, the arabo-xylan which was recovered, had an unchanged xylose/arabinose ratio, (2.5:1) a 4% glucosan content, and a specific rotation of -122°. Assuming the remaining glucosan to be β-glucosan, the rotation of pure pentosan is calculated as -128°, slightly more positive than the values quoted for the water-soluble pentosans of rye and wheat. The small difference might be accounted for by the continued presence of some starchy glucosan (but this seems unlikely) or possibly by the smaller proportion of arabinose residues present in the molecule.

Gum - hemicellulose relationships

The idea is now well-established that the division between the gums and the hemicellulose, extracted by dilute alkali, is somewhat artificial. For example, investigation shows that a β-glucosan is extracted by dilute alkali which appears to resemble very closely the initially water-soluble

β -glucosan. The preparations obtained from Bromus mollis all contain glucosan, and the fact that extraction by means of autoclave treatment gives β -glucosan, clearly very similar to that extracted by water at 40°C., is further support for the view that solubility differences do not necessarily reflect a difference in the fundamental nature of the molecule. In fact, all hemicellulose preparations were rendered water-soluble as a result of the soda extraction, indicating that their resistance to water extraction may be due to some encrusting material rather than to actual solubility.

Enzymic degradation of the autoclave extracted Bromus mollis preparation, which is predominantly glucosan, was carried out by use of an enzyme prepared from kilned malt. In addition to glucose, laminaribiose, laminaritriose and other oligosaccharides were chromatographically detected. Cellobiose was not detected. Treatment with a green malt enzyme of the same preparation and also of the gum and hemicellulose fractions of Bromus mollis, precipitated by acetone after addition of Fehling's solution, produced in each case those same oligosaccharides, along with a disaccharide with the mobility of cellobiose (some of this, particularly from the gum fraction may be maltose). The oligosaccharides produced depend on the enzyme used, but those

degradations help to illustrate the similarity of the three preparations and demonstrate the presence of both 1,3 and 1,4 linkages in each (Plate 6).

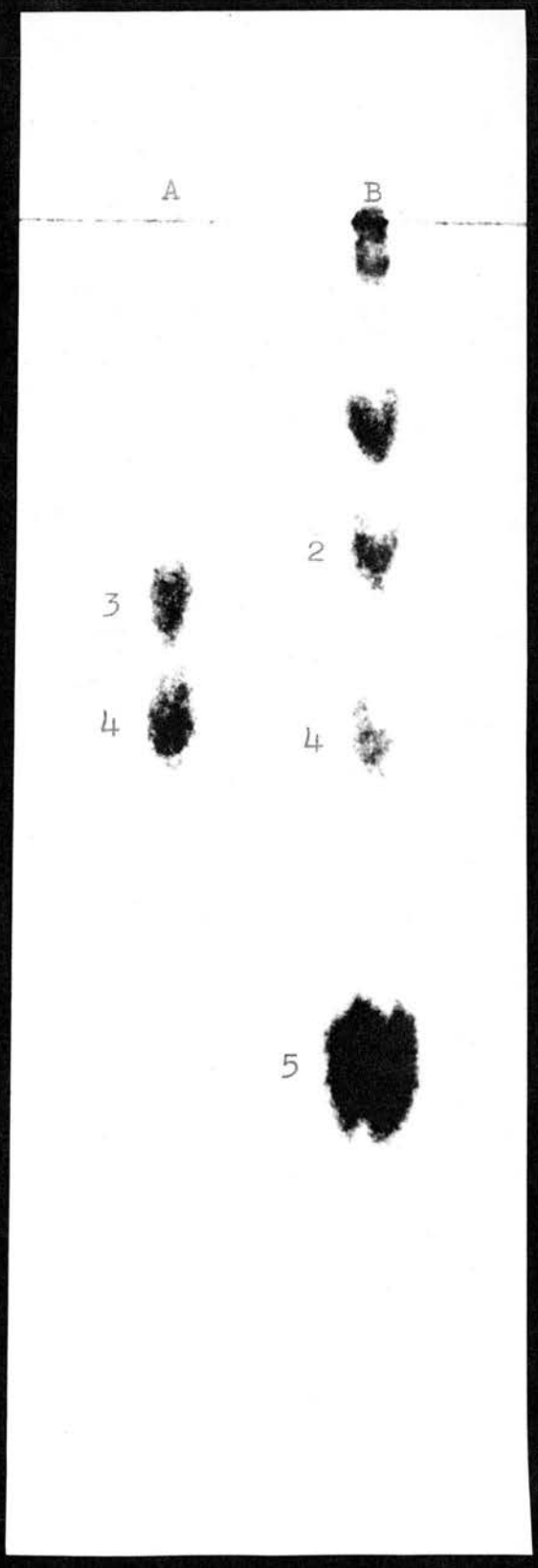
Although the water-extracted and soda-extracted pentosans also show close similarity to each other, it is necessary to draw some distinction in considering them. Preece & Hobkirk (1954) distinguish between endospermic and husk-type hemicelluloses, and it would appear that water-extracted pentosan, in which uronic acids are not detected, is substantially of the endospermic type. Of the soda-extracted pentosan, the husk-type is distinguished by containing uronic acid residues, and apparently by a high xylose/arabinose ratio. It is observed that wild oats seeds, with a high husk content, gave a high yield of pentose hemicelluloses, with a high xylose/arabinose ratio, and containing uronic acid residues.

Reference has been made to the wide variety existing in the content of water-soluble preparations, and those contrasts are accentuated on consideration of the hemicelluloses. Bromus is clearly distinguished by its high content of "hemicellulose", both water- and soda-extracted and consisting of both β-glucosan and pentosan, while in contrast canary seed contains no gums and a relatively small quantity of hemicelluloses. Furthermore, wild oats (which closely resembles cultivated oats) has a high content of

water-extracted β-glucosan, while soda-extraction produces almost pure pentosan, most of which is believed to be obtained from the husk.

To account for such fundamental differences in content, requires a more exact knowledge than is available at present of the location of those hemicelluloses and of their precise functions. There appears to be sufficient evidence to state with certainty that such materials serve as structural polysaccharides and in this connection, examination of several seeds shows that there is a vast difference in the thickness of endospermic cell-walls and that the cell-walls of all the Bromus seeds are unusually thick (Plate 7). It is also likely that those carbohydrates are utilised as substrates for synthesis and respiration during germination of the seeds, but an explanation of the presence of different polysaccharides in different seeds of the grass family is still awaited.

PLATE VI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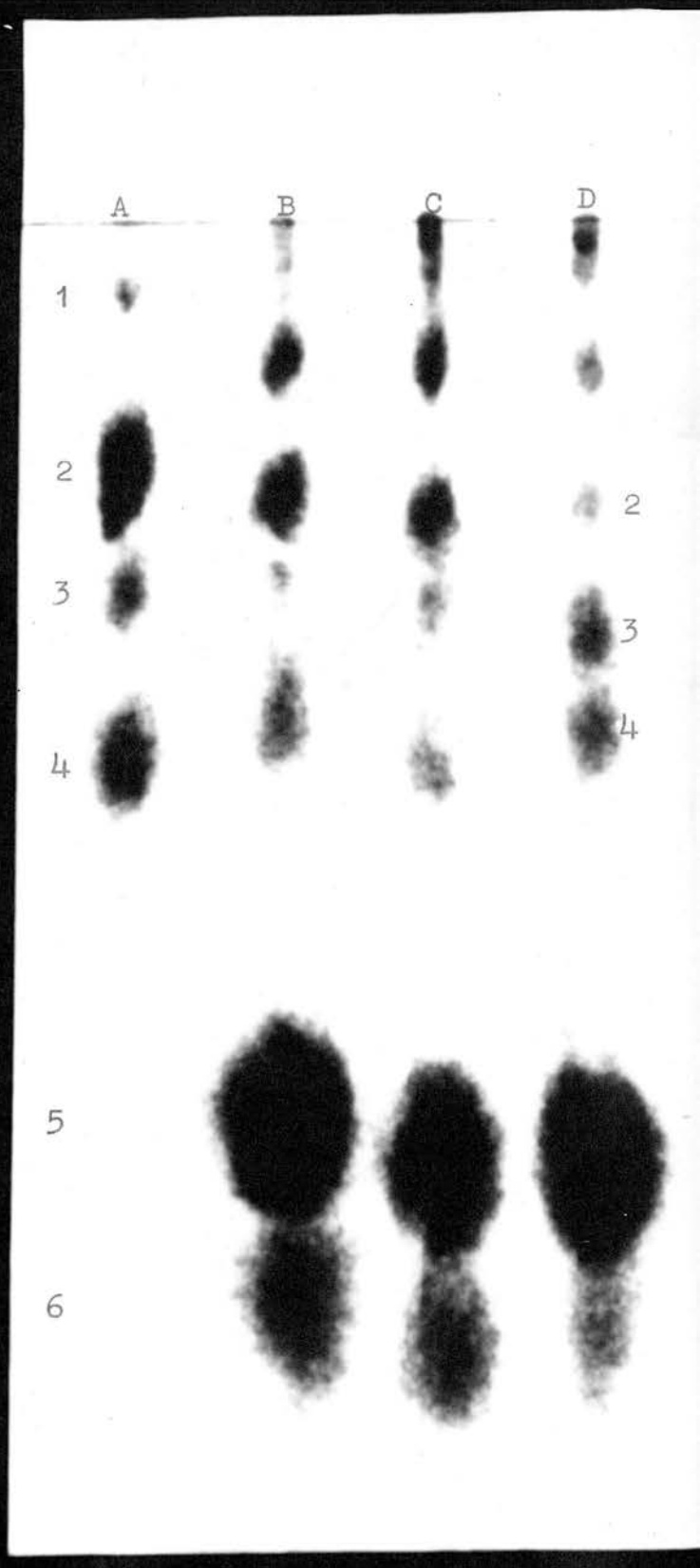
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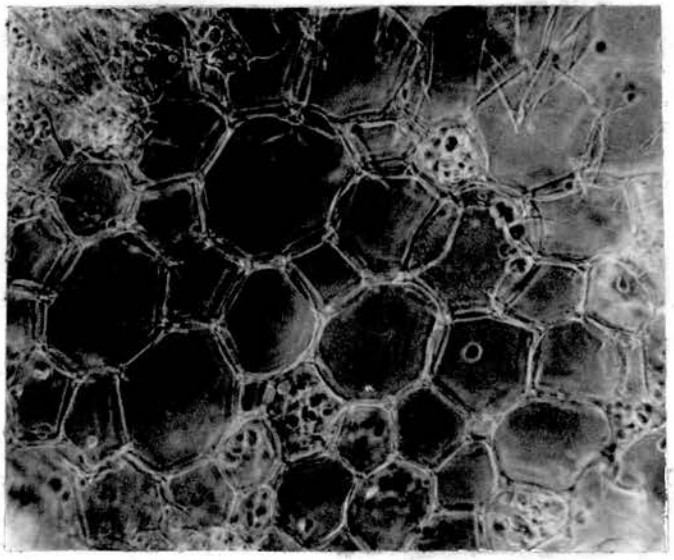
PLATE VII



SECTION III

THE TAXONOMY OF THE GRAMINEAE

The Taxonomy of the Gramineae

Considered as an entity, the Gramineae is a distinctive and well-defined family of flowering plants. There is some disagreement, however, as to the most appropriate allocation of genera to different tribes within the family although the present trends in classifying the grasses appear to be reflected in the creation of a multiplicity of individual tribes. Thus Haeckel (1887) assigned the British genera of the Gramineae to only 8 different tribes, while Clapham, Tutin & Warburg (1952) refer these genera to 15 and Hubbard (1954) to no fewer than 19 separate tribes.

The primary reason for such botanical and in fact all biological classification was one of convenience; it became essential to develop some sort of arrangement for the identification of plants as increasing numbers proved to be of economic importance and became subjects of investigation. An "alpha" taxonomy was developed, a classification based mainly on morphological grounds. A distinguished example of such a classification was that of Linnaeus, which was based on the habit or on the number of the flower parts. Such a system is "artificial" in as much as it depends on a minimum of botanical characteristics without regard to any "natural" grouping of related plants. By including more

characters, the taxonomist is gradually approaching an "omega" taxonomy - a completely "natural" classification. It will be observed that in this context the difference in meaning between "artificial" and "natural" is a difference of degree. This trend, which seeks to include a maximum number of characters to assist the classification has caused investigation of other than purely morphological features. Those include such characters as the nature of the starch grains, and probably the most important and fundamental of all, consideration of chromosome size and number.

Little attention has been accorded in the past to the possible application of chemical composition to taxonomic problems. With regard to the Gramineae, the study of starches by Reichert (1919) and the work of de Cugnac (1931) probably represent the only systematic attempts of this kind. De Cugnac investigated a wide range of grasses and divided them into two groups depending on the sugar content of their vegetative parts. From the taxonomist's point of view such an investigation could be of little systematic value, but it serves to demonstrate the potentialities of such an approach. Although some authorities dismiss the possibility of considering chemical composition for taxonomic purposes, Lawrence (1951) has suggested that many of the findings of plant

biochemists have received too little attention from taxonomists. He considered that further biochemical studies of the flowering plants may eventually be of major importance in confirming or rejecting the transfer of genera from one taxon to another. It is clear that a purely artificial classification of plants of widely different families might arise on the basis of the presence or absence of a particular chemical substance or group of substances and such an approach would of course be extremely misleading. The value of chemical composition in such considerations appears to be summarised by Turrill (1938) in those words, "Within limited groups of plants a character may aid in classifying species..... applicability in closely related groups of plants is not to be belittled by the fact that the same substance may occur in very distantly related plant families".

Although the first and most obvious purpose of taxonomy was one of convenience, it is important to observe its probable phylogenetic implications. With regard to carbohydrate content of the grass seeds, it seems likely that the differing contents detected in different seeds at ripeness are the result of the various enzyme systems prevailing in each seed. In turn, those enzyme systems must presumably be gene-controlled so that it is probable that the amount of various carbohydrate fractions present at ripeness must

depend on the genetic constitution of the plant. Whether the carbohydrate content of the seed is as invariable as certain of the floristic characteristics is a question only to be answered completely by the amassing of fuller analytical data.

Discussion of Results from Sections I and II

Table 16 contains lists of the tribes to which authorities have ascribed various British grass genera. Some important re-arrangements as a result of more recent investigations have occurred, e.g. Nardus which had been considered a member of the Hordeae is now ejected from the tribe because of differences in starch grain. Similarly Brachypodium which had hovered between Festuceae and Hordeae has been found to possess chromosomes which are strikingly different from those of the members of either of these tribes.

Tables 17 and 18 show the groupings into which the grasses, now investigated, are placed on the basis of sugar and water-soluble polysaccharide contents. Six groups have been distinguished by consideration of the sugars, those arising from the four groups shown in Plate 5 by distinguishing some further small but distinct differences. This grouping, depending as it does on the qualitative detection of sugars, is clearly rather more convincing than that based on polysaccharide

content which requires consideration of relative amounts of the molecular entities present.

Reference to Table 3 and to Table 1 in which the classification of Hubbard (1954) is adhered to, shows that two tribes (as represented by the members now examined) are uniquely characterised by the sugar content of their seeds. These are the Bromeae, containing fructosans but lacking raffinose and the Hordeae containing both fructosans and raffinose. Three species of the Bromeae now examined and a fourth, B. erectus, examined by another worker, adhered to this generality and although only two members of the Hordeae, Agropyron and Elymus, are now investigated, the results available for the cultivated members of this tribe, wheat, oats and barley, also conform to this distinction. Spartina containing as it does no oligosaccharide more complex than sucrose, would appear to be fully characterised by its sugar content but as already observed, the absence of any true resting-stage in the seeds of this grass makes any statement concerning the sugar content of its "seeds" of doubtful value.

All members of the Aveneae and Agrostideae examined exhibited the commonest pattern of sugar distribution (hexose, sucrose, raffinose and stachyose) as did Brachypodium and three of the Festuceae (Poa, Dactylis and Cynosurus). Clearly, in its content of

free sugars and also in the nature of the water-soluble polysaccharides present (Table 7) Brachypodium is quite distinct from Bromus, though Clapham et al. (1952) assign both to the tribe Brachypodieae.

Festuceae is the only tribe whose members are distributed between two groups on the basis of their contents of sugars; Festuca and Lolium are again uniquely characterised by containing the "raffinose isomer" trisaccharide, and it is interesting that Hubbard (1948) has already suggested a link between those genera on botanical grounds. In the past the Festuceae harboured many genera which have now been evicted from the tribe (e.g. Phragmites, Sieglingia, Molinia, Bromus, Brachypodium and Glyceria) and these refugee members have now (Hubbard, 1954) been assigned to different, isolated tribes. Hubbard (1948) regards the Festuceae as the most ancient tribe of the group of mainly temperate-climate grasses, and it may well be that, despite the eviction of some of the obviously less desirable members, the residue is still rather heterogeneous with regard to both biochemistry and floral morphology.

The species comprising Group 4 of Table 17 (characterised by the apparent absence of oligosaccharides more complex than raffinose) belong to four separate rather isolated tribes, three of which (Nardeae, Danthonieae and Glycerieae) are not clearly

related to other well-authenticated tribes. Except in so far as it confirms the fact that Nardus does not show any close affinities to the Hordeae, the sugar content of the seeds offers no useful clues to the possible relationships of these tribes.

With regard to the water-soluble polysaccharides, (Table 18) it is clear that Nardus (Nardeae) and Molinia (Danthonieae) are uniquely characterised by their possession of, respectively, very high concentrations of mannan and galactan. Molinia is tentatively assigned to the Danthonieae by Hubbard (1948) along with Sieglingia which it resembles in certain morphological features of the leaf, and in basic chromosome number; it differs from Sieglingia in the rather high content of galactan, though it resembles it in the general pattern of sugar distribution. Certainly the water-soluble carbohydrates of Molinia show no relationship of this genus with the Festuceae, with which it was formerly classed.

The grasses whose seeds are rich in β-glucosan belong to the Bromeae, the Festuceae, or the Aveneae. The Bromeae have already been shown to constitute a very distinctive group by virtue of their content of sugars and oligosaccharides. In the Festuceae, Dactylis and Festuca contain large amounts of β-glucosan, while Cynosurus, Poa and Lolium contain

very much smaller quantities of this material. Only two of the Aveneae (Avena and Arrhenatherum) contained typical β-glucosan; it is of interest to note that the two genera which lack β-glucosan have somewhat uncertain affinities with this tribe. Thus Hubbard (1948) states that Holcus has no obvious near relatives in the Aveneae, while Clapham et al. (1952) assign Anthoxanthum to the Phalarideae.

With the exception of Festuca, the seeds with pentosan-rich polysaccharides all belong to the Bromeae or Hordeae. The cultivated cereals from the Hordeae all contain relatively high amounts of water-soluble pentosan within their seeds; barley is somewhat anomalous in being the only member of this tribe to contain a large amount of β-glucosan. On the basis of their content of water-soluble polysaccharides, it would appear that Elymus and Agropyron have rather strong affinities with rye and wheat, while barley occupies a more isolated position; from the point of view of sugar content, on the other hand, the tribe appears to be a homogeneous one.

In summarizing the taxonomic implications of this study of the water-soluble carbohydrates of seeds of the Gramineae, the following points may be considered of particular significance:

1. Nardus occupies an isolated position, by virtue of its high content of water-soluble mannan.

2. The Bromeae form a very natural tribe of genera, quite distinct from the Brachypodieae the Festuceae and the Hordeae.

3. The Hordeae form a natural tribe, as revealed by their sugar content; on the basis of their content of soluble polysaccharides, Hordeum itself is rather distinct from the other genera examined.

4. All the Festuceae contain soluble β-glucosan in greater or lesser amounts, but two genera (Lolium and Festuca) are distinctive in containing an unusual trisaccharide.

5. Two of the Aveneae, as recognised by Hubbard (1948) differ from the other two (Avena and Arrhenatherum) in completely lacking β-glucosan. The inclusion of these two genera Anthoxanthum and Holcus in the Aveneae, is slightly suspect on morphological grounds; in their contents of soluble carbohydrates, both show affinities with the Agrostideae and, to a lesser extent, with Phalaris.

In conclusion, the preliminary nature of this study must be emphasised, but nevertheless it is interesting to note that, using the criteria outlined above and ignoring all morphological data, over half of the species examined could be distinguished absolutely by their contents of water-soluble carbohydrates. This material normally represents rather less than 5% of the dry weight of the seeds,

and it may be suggested that a detailed study of other components might yield results of far-reaching taxonomic importance.

Table 16

Classifications of the Gramineae

Genus	Tribe			
	Engler & Prantl (1887)	Hubbard (1948)	Clapham et al. (1952)	Hubbard (1954)
Sieglingia	Festuceae	Danthonieae	Danthonieae	Danthonieae
Molinia	Festuceae	Danthonieae	Danthonieae	Danthonieae
Festuca, Poa, Dactylis, Cynosurus	Festuceae	Festuceae	Festuceae	Festuceae
Lolium	Hordeae	Festuceae	Festuceae	Festuceae
Bromus	Festuceae	Festuceae	Brachypodieae	Bromeae
Brachypodium	Festuceae	Festuceae	Brachypodieae	Brachypodieae
Glyceria	Festuceae	Festuceae	Glycerieae	Glycerieae
Avena, Arrhenatherum, Holcus	Aveneae	Aveneae	Aveneae	Aveneae
Agrostis, Ammophila, Phleum	Agrostideae	Agrostideae	Agrostideae	Agrostideae
Anthoxanthum	Phalarideae	Phalarideae	Phalarideae	Aveneae
Phalaris	Phalarideae	Phalarideae	Phalarideae	Phalarideae
Nardus	Hordeae	Nardeae	Nardeae	Nardeae
Agropyron, Elymus	Hordeae	Hordeae	Hordeae	Hordeae
Spartina	Chlorideae	Chlorideae	Spartineae	Spartineae
Setaria	Paniceae	Paniceae	Paniceae	Paniceae

The genera are arranged according to Clapham et al. (1952)

Table 17

Grass Seeds Grouped According to Sugars Content

Sugars Present		Genera in Group (Species as in Table 1)
Group 1	Hexoses and sucrose only	Spartina
Group 2	Hexoses, sucrose and homologous series of fructosans	Bromus
Group 3	Hexoses, sucrose, homologous series of fructosans and raffinose	Elymus, Agropyron
Group 4	Hexoses, sucrose and raffinose	Glyceria Phalaris Nardus Molinia
Group 5	Hexoses, sucrose, raffinose ans stachyose	Brachypodium Poa, Dactylis, Cynosurus Arrhenatherum, Avena, Holcus, Anthoxanthum Ammophila, Agrostis, Phleum, Setaria
Group 6	Hexoses, sucrose and isomer of raffinose	Festuca, Lolium

Table 18

Grass seeds grouped according to nature of water-soluble polysaccharides present.

Distinguishing characteristic	Genera in group (species as in Table 1)
Group 1 β-glucosan present	Bromus Arrhenatherum, Avena Dactylis, Festuca, Poa*, Lolium* Cynosurus*
Group 2 Mannan approximately 20% of polysaccharide	Nardus
Group 3 Galactan more than 20% of polysaccharide	Molinia
Group 4 Pentosan more than 20% of polysaccharide	Brachypodium Agropyron, Elymus Glyceria Sieglingia
Group 5 α-glucosan more than 80% of polysaccharide	Phalaris Holcus, Anthoxanthum Ammophila, Agrostis, Phleum Spartina, Setaria

* Minor constituent of crude polysaccharide.

SECTION IV

CARBOHYDRATE CHANGES DURING GERMINATION

INTRODUCTION

The profound changes which the carbohydrates of seeds undergo on germination has already been referred to in Sections I and II. Investigations, which have been carried out almost exclusively on barley, have provided information with regard to three distinct molecular groups of carbohydrates, namely the sugars and hemicelluloses, already referred to, and starch (Aspinall, Hirst & McArthur, 1955).

During the systematic study of the carbohydrate content of the Gramineae, which has been undertaken, perhaps the two most striking observations made were the detection of a "raffinose-type" trisaccharide in Lolium and Festuca and the very high gum and hemicellulose content of Bromus seeds. It was thought desirable to carry out investigations as to the fate of those carbohydrates on germination. In particular it was hoped to compare the changes in the "raffinose isomer" content to the spectacular utilisation of raffinose in barley. With regard to Bromus seeds, their high content of not only β-glucosan but also arabo-xylan material presents the opportunity of obtaining some indication of pentosan changes on germination.

It should be stressed that the practical difficulties involved in a laboratory germination of relatively small seeds were considerable, the most

restricting being the lack of uniformity of growth and incomplete germination. In those circumstances the results which have been obtained, while indicating the general trends, cannot be regarded as precise reflections of the changes which occur in seeds germinating in the field.

Sugar Changes during the Germination of the Seeds of Lolium perenne

10 g. samples of seeds were germinated between sheets of blotting paper in an incubator in the dark at 27°C. This rather high temperature, while perhaps leading to some complications, was used on the recommendation of officials of the Scottish Seed Testing Station, East Craigs, Edinburgh, so that almost complete germination was obtained. For germination a glass plate, 11 inches square and with a small circular hole at the centre, was placed on a glass trough full of water. The seeds were spread on a sheet of blotting paper which was laid on the glass plate and which was kept damp by means of a wick at its centre dipping into the water of the reservoir. Rootlets were apparent after two days and after four days the coleoptiles were approximately $\frac{1}{3}$ inch long.

After periods of two and four days, seed samples were plunged into boiling 80% ethanol and refluxed for two periods of 45 minutes to inactivate enzymes and

Table 19

Sugars of Lolium seeds during germination

(mg. sugar, as glucose equivalent per 100 g. seed*)

Period of Germination	0 days	2 days	4 days
Raffinose isomer	1360	568	374
Sucrose	577	878	2045
Glucose	147	195	2075
Fructose	149	112	1438
Maltose	-	+	+
Higher oligosaccharides	-	-	+

*Seeds weighed before germination

+Detected chromatographically

-Not detected.

extract some of the sugar. The seeds were then dried at room temperature, ground and the remaining sugar extracted by again refluxing in alcohol. The sugars were then estimated after chromatographic separation by the methods already described in Section I, except that 10 mg. ribose was "weighed in" to each extract as a reference sugar. The relative and absolute quantities of sugar were calculated first as ribose and finally as glucose equivalents. The "weighing in" technique was preferred to the previously employed method which first determines total reducing sugars because the presence of reducing oligosaccharides of the maltose type considerably complicates this method.

Discussion of Results

Three seed samples were investigated, namely ungerminated seeds and seeds after two and four days growth. Table 19 contains details of the quantities detected of the "raffinose isomer", sucrose, glucose and fructose.

The most significant change was the spectacular drop in concentration of the "raffinose isomer". This sugar occurred in a relatively high concentration and it appears that most of it was utilised in the early stages of growth. In this respect it is similar to raffinose in barley (James, 1940; MacLeod, 1957). It will be observed, however, that a considerable part of it persisted even after four days. In this

connection, the results of MacLeod (1957) show that the concentration of raffinose drops only very slightly in germinating barley which has previously been anaerobically steeped, and a similar effect might explain the persistence of some "raffinose isomer" in Lolium, since a proportion of those small seeds must certainly be in very close contact with the damp blotting paper, and perhaps undergo a treatment with an effect similar to anaerobic steeping. While the "raffinose isomer" concentration had dropped very considerably, it will be observed that there was a large increase in both sucrose and monosaccharide content, the increases in those sugars being similar to values quoted for sugar changes in a laboratory germination of barley (MacLeod et al., 1953). Maltose and the higher maltose-type oligosaccharides were detected on germination but no free galactose could be detected in spite of the considerable utilisation of the "raffinose isomer". It is not, of course, certain that the metabolic pattern of the disappearance of the "raffinose isomer" is the same as that of raffinose in barley, but those restricted observations suggest close similarities.

Polysaccharide Changes during the Germination of the Seeds of Bromus mollis

Preliminary investigation was carried out to determine the most satisfactory conditions of

germination. At 20°C., not more than 60% of the seeds germinated. Germination at 25°C. improved this figure considerably and a further slight improvement was obtained by first dehusking the seeds by use of sulphuric acid (Pollock, Essery & Kirsop, 1955). This dehusking treatment furthermore allowed a more uniform germination. 20 g. seed samples (weighed before dehusking) were used, germinations being carried out at 25°C., and otherwise under the same conditions as described for the seeds of Lolium. In those conditions, almost exactly 75% of the seeds of each sample germinated. It is at once conceded that this incomplete germination complicates consideration of the results obtained and perhaps somewhat reduces their value, but the implications of this will be discussed later. Although growth was not uniform, in general the first rootlets emerged after two days, and after four days coleoptiles had emerged from at least 60% of the seeds and were approximately ¼ inch long. Between four and six days, there was considerable growth and another 15% of the seeds germinated. After eight days, the shoots were about 1 inch long.

At two day intervals, samples were removed from the incubator and refluxed in 80% ethanol before and after grinding, as described for the rye-grass seeds. In this instance, care was taken to grind the seeds

fairly finely, each sample being treated in exactly the same way. After the alcoholic extraction, successive extractions were carried out by methods already described in Section II, to obtain the gums, soluble in water at 40°C., the non-starchy polysaccharide rendered soluble in water by autoclave treatment, and finally the hemicelluloses soluble in 4% sodium hydroxide. Throughout extraction and precipitation, precautions were taken to ensure that conditions were as reproducible as possible; in particular the temperature of the aqueous extraction was controlled very closely at 40°C., and precipitations were carried out at 10°C. Precipitates formed on addition of Fehling's Solution alone were not removed before subsequent addition of acetone.

Preparations were washed, taken to dryness and weighed. 20 mg. portions were hydrolysed and the proportion of the constituent sugar units determined.

Before giving consideration to the results obtained the implications of the preliminary treatment must not be ignored. The obvious risk must be considered, that the samples (which were dehusked separately) might be affected differently by the acid treatment. With regard to this problem, the treatment was, as far as could be ascertained, reproducible, in each case 20 g. samples of the seeds being immersed in 250 ml. 50% H_2SO_4 at 10°C. and stirred gently for $3\frac{1}{2}$ hours.

By inspection of the seeds, it appeared that a constant amount of outer integument was removed leaving the embryo and endosperm enclosed in a leathery testa. It is thought, therefore, that the seeds in each sample underwent an approximately equivalent treatment. It will be seen (Table 20) that a small increase in araban content was obtained after dehusking. This increase, although small, seems rather too great to be ignored and can only be accounted for by assuming that the preliminary treatment resulted in extraction of a small amount of material not previously extracted. In other respects, the effect of dehusking was, as to be expected, the removal of some pentosan and also of a certain amount of glucosan. It will be observed, however, that the yields of material extracted by water at 40°C. before and after dehusking were very similar. The obvious advantage of dehusking, apart from the fact that it gave purer products, is that it allows easier investigation of the endosperm of the seed, in which the main metabolic processes involving seed polysaccharides are presumed to occur.

Results

The three groups of polysaccharides investigated were those obtained by extractional processes of increasing severity, but it is important to realize that although 4% NaOH would be expected to render

Table 20

Yields of Preparations

(mg. anhydrosugar units per 100 g. dry seed)

Preparation	Total	Glucosan*	Xylan	Araban
(Dehusked) Days Germinated				
40°C. Gum				
0 Days	1176	754 (520)	306	118
2 Days	2023	1337 (467)	383	303
4 Days	2340	1684 (724)	492	164
6 Days	1576	946 (189)	488	142
8 Days	1348	728 (124)	364	256
Autoclave Gum				
0 Days	6023	3856	1325	842
2 Days	8821	6174	1676	971
4 Days	7753	5582	1628	543
6 Days	5528	3317	1714	497
8 Days	3340	2070	736	534
Hemicellulose				
0 Days	5204	2185	1874	1145
2 Days	5316	1967	2552	797
4 Days	5175	1501	3002	672
6 Days	4416	884	3046	486
8 Days	3190	797	1946	447
Ungerminated (with husk)				
40°C. Gum	1295	720	325	250
Autoclave Gum	7260	5228	1379	653
Hemicellulose	7750	3290	3540	920

*Figures in brackets are approximate β-glucosan contents

Table 21

Carbohydrates extracted from seeds of

Bromus mollis (% of dry seed)

Days Germinated	Total Recovered*	Total Unrecovered†
0	12.40	16.81
2	16.16	12.56
4	15.27	13.13
6	11.52	6.82
8	7.88	8.85

* From Table 20; sum of 40°C. gum, autoclave gum and hemicellulose.

† Mainly starch, lost by dialysis after amylase treatment of autoclave extract (estimated by Somogyi reagent after hydrolysis).

FIGURE 3

GERMINATION OF BROMUS SEEDS.

YIELDS OF β GLUCOSAN (Mg. per 100 g. seed)

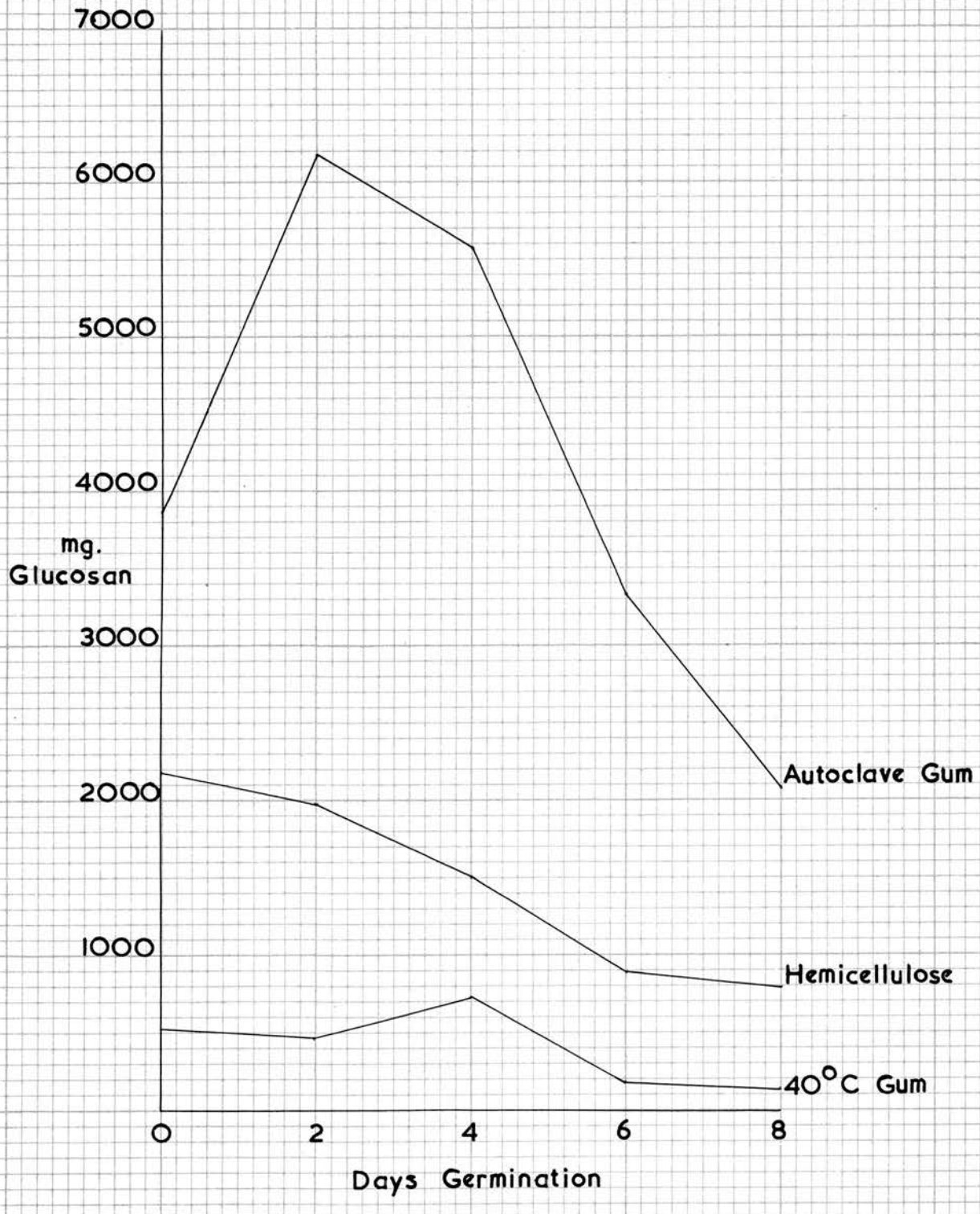


FIGURE 4.

GERMINATION OF BROMUS SEEDS.

YIELDS OF XYLAN (Mg. per 100 g. seed)

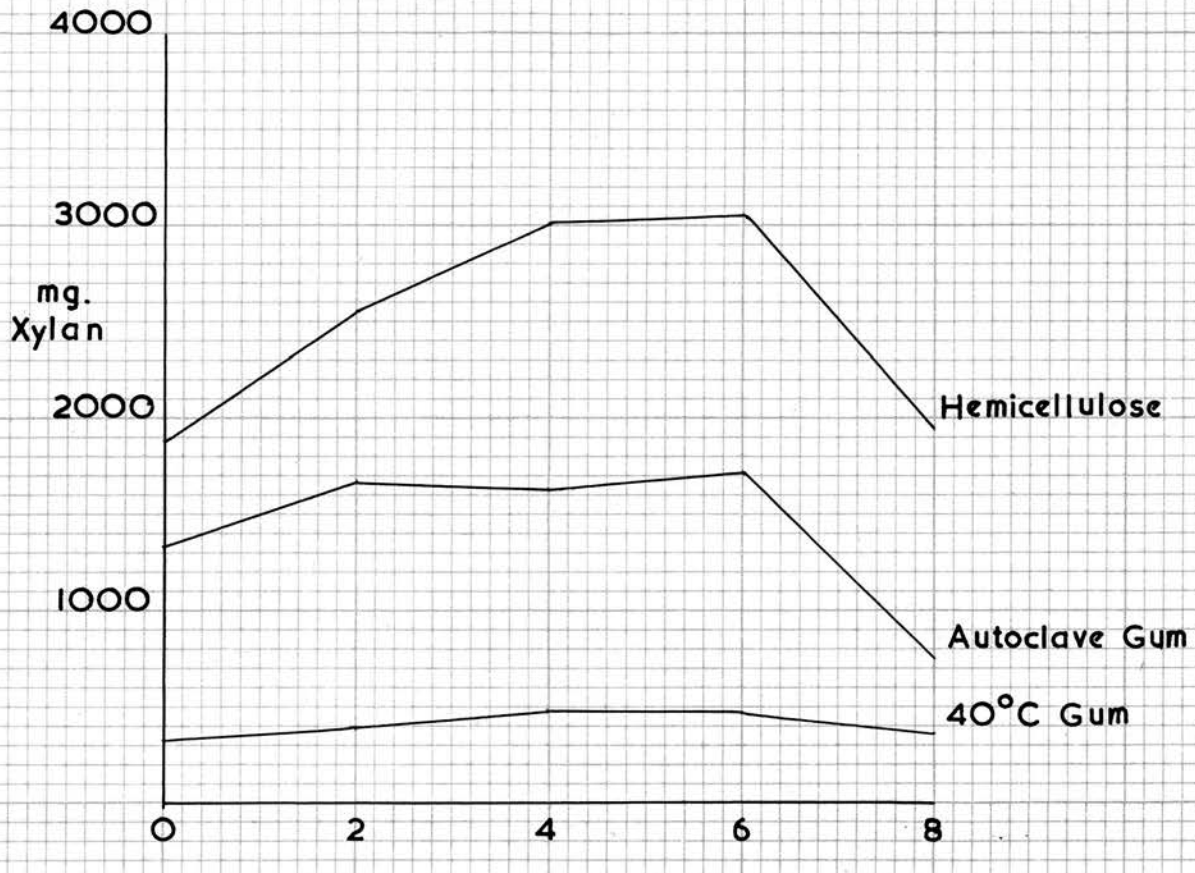
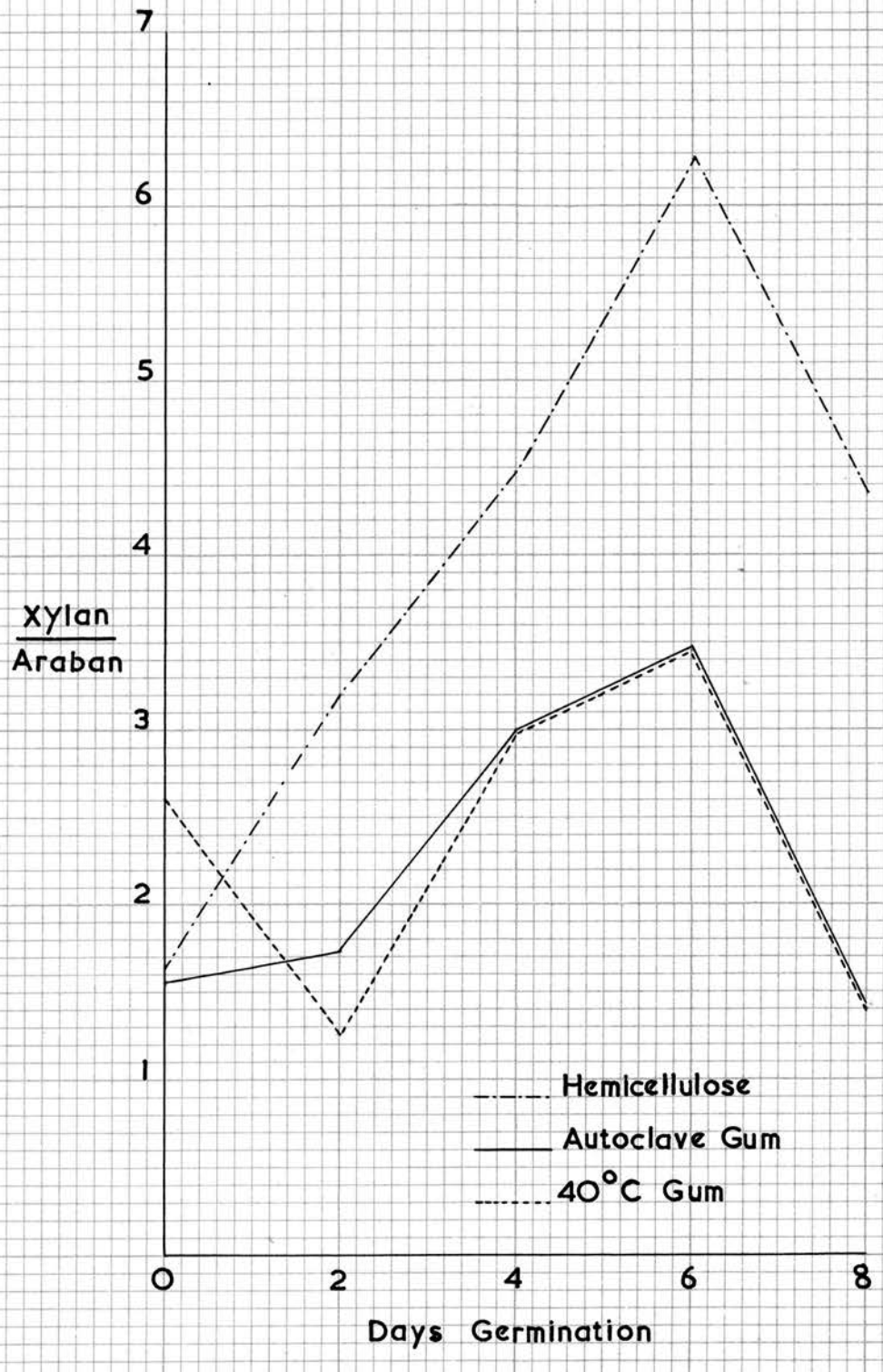


FIGURE 5.

GERMINATION OF BROMUS SEEDS.

RATIO OF YIELDS :- XYLAN TO ARABAN.



soluble much of the endospermic polysaccharide, there is a considerable reserve, more resistant in nature and only extracted either by use of a stronger reagent or by previously rendering more soluble by enzymic or mechanical means. The seeds of all the species of Bromus examined were characterised by their possessing unusually thick endospermic cell-walls, a fact which is presumably reflected in the unusually high gum and hemicellulose content. It is likely that those thick-walled seeds would require extractional treatment more exhaustive than usual to effect complete solubilisation of the cell-wall carbohydrates.

As to the changes in water-soluble carbohydrates and hemicelluloses during germination, once more the limitations and preliminary nature of the investigation must be emphasised. Samples at two day intervals over a total of eight days were used, with the result that while a broad picture was obtained, it was possible that the relatively long intervals between sampling resulted in failure to detect rapid changes. Furthermore, germination occurred only in 75% of the seeds, and was somewhat uneven. The yields and compositions obtained could not therefore represent the exact circumstances if germination had been complete. An approximate correction for incomplete germination could be made by

assuming that each preparation contained 25% of the carbohydrate content of preparations obtained from ungerminated seeds. This course, however, is open to objection as autolytic changes must occur in the moist, ungerminated seeds, and there is no doubt that the figures reported succeed in reflecting the trends of the major changes. Yields as anhydro sugar unit content of each fraction are quoted in Table 20; the total yields obtained from all extractions are presented in Table 21. Table 21 also contains figures representing the percentage of polysaccharides extracted by the autoclave treatment but not recovered after amylolysis and dialysis. Those figures therefore approximate to the starch content of seeds, except perhaps in the last sample when considerable synthesis of polysaccharides must have occurred in the shoots. In Table 20, the figures in brackets in the glucosan column of the gums represent the approximate content of β-glucosan, the remainder being α-glucosan, starchy in nature. Those figures were obtained by calculation from the specific rotation of the preparations, on the assumption that the pentosan has specific rotation of -135°, the Bromus β-glucosan -9°, and the starchy glucosan approximately $+185^{\circ}$. Results are also shown in figures 3, 4 and 5.

After two days the most spectacular change

occurred in the glucosan content of the autoclave-extracted gum. This represented an increase in that fraction of over 2000 mg./100 g. seed, an increase which was not accompanied by any notable change in the glucosan of the gums extracted at 40°C. or of the hemicelluloses. At later stages, apart from a slight increase in the gum (40°C.) at four days, β-glucosan content in each fraction decreased very markedly until after eight days, β-glucosan of the 40°C. gum (making allowance for incomplete germination) had dropped virtually to zero. Pentosan changes are perhaps best reflected by the xylan content since total araban may include some free araban. No rise occurred as spectacular as the early increase in the autoclave-extracted glucosan but after two days each fraction had increased in xylan content. This increased yield became even higher until between six and eight days there was a remarkably rapid drop, the total extracted from the three fractions at eight days being somewhat less than that obtained from ungerminated seeds. The most notable changes occurred in the soda-extracted fraction, rather than the autoclave-extracted fraction as in the case of glucosan, and the initial increases were not so rapid. The ratio of xylan/araban generally decreased during germination until the 8th day when there was a relative increase in araban content. This may be

accounted for by free araban in the shoots. The ratios for 40°C. gum and autoclave-extracted gum were almost the same, indicating the similarity and perhaps identity of the pentosans in those two groups.

Discussion

It is perhaps more convenient to consider first the later stages. The drop in glucosan in those stages is consistent with the results already available for malting barley (Preece & Hoggan, 1957) which show that β-glucosan extracted by water and dilute soda disappears; furthermore the drop in pentosan content also agrees with their observations. β-glucosan content began to fall rapidly at about four days, or in fact at about the time when the starchy content fell considerably, and when a fairly rapid change was observed visually in the content of the seeds. Pentosans on the other hand, were slower to decrease. It seems safe to postulate that glucosanase and presumably less active pentosanase enzyme systems caused those decreases. It would further appear that the glucosanase system was sufficiently active to prevent any accumulation of β-glucosan in the 40°C. fraction. The rapid drop of soda-extracted glucosan indicates that glucosan reserves of this nature were completely mobilised.

With regard to the changes occurring in the earlier stages of germination, both glucosan and

pentosan content increased at the second day and pentosan content further increased till the sixth day. The change of greatest magnitude occurred in the glucosan content of the autoclave-extracted polysaccharide after two days, and any satisfactory explanation of the total changes must include consideration of this increase. It should be noted that this preparation was virtually pure carbohydrate and its specific rotation of -37^{o} seems to preclude the possible presence of starchy material. It will be borne in mind that the material extracted by the treatments used, does not represent the total carbohydrate content of the seed, and that the increase in material extracted during the early stages of germination will be accounted for by solubilisation of some of this more resistant polysaccharide. It seems that the large glucosan content must be accounted for by the initially soda-soluble material becoming more soluble and by more resistant material becoming soluble in dilute soda. It first suggests itself that this increase might be accounted for on purely mechanical grounds, but in those circumstances, the resistant pentosan, which almost certainly is present, should equally easily be solubilised. There is in fact a large increase in pentosan content but this does not take place so rapidly. It therefore seems possible to explain

those observations only partly on the basis of a mechanical solubilisation, and an enzymic solubilising effect, more active with respect to the glucosan than to pentosan, seems the likely explanation of part at least of those changes.

Very striking changes have been shown to occur during the germination of the seeds and it is interesting to note that the endospermic cells tend to disintegrate easily even after simply steeping the whole seed in water. This observation seems to support the concept of enzymic solubilisation of the cell-walls. From two till eight days more than 8% of the seed weight, as non-starchy polysaccharide disappears. 75% of this degraded polysaccharide is β-glucosan and when it is remembered that the starch content does not exceed approximately 15% it becomes clear that this change in hemicellulosic content is very considerable. It is not possible to state definitely that those hemicelluloses are in fact used as a substrate in the early stages of growth, but there is no doubt that the circumstantial evidence points towards such a utilisation, especially in respect of the β-glucosan.

It may be suggested that seeds of Bromus would yield a useful means of studying pentosan enzymolysis. The fall in xylan is most marked after six days growth, and the initially high pentosan content might allow changes to be more readily detected than is the case with cereal seeds.

GENERAL DISCUSSION

GENERAL DISCUSSION

It is to be reiterated that the molecular entities included under the title "Water-soluble Carbohydrates" are not the constituents of a homogeneous group. The somewhat artificial restriction imposed by virtue of their water-solubility is a restriction primarily of convenience, which is inevitable in undertaking a relatively wide survey. Nevertheless a general review of the work accomplished provides the opportunity to consider any relationship which might exist among the carbohydrates included within this definition, and thereafter to discuss the wider question, already referred to in Section II, as to whether any relationship exists between those carbohydrates and others present in the seeds.

The sugars and oligosaccharides which are extracted by alcohol and which, with the exception of the fructosans, are largely located in the seed embryo, serve by mechanisms imperfectly understood, as substrates for the early stages of germination. While indirect evidence is available to suggest that the gum-like polysaccharides are utilised in the growth of the seedlings at a later stage, it is reasonably certain that their function is primarily a structural one. There is therefore a vast difference in both structure and functions of those two groups of water-

soluble polysaccharides, and any relationship between them would seem unlikely. This point of view appears to be upheld in the case of the low molecular oligosaccharides of the "raffinose family". The most complex member of this family contains only five sugar units and the evidence available is strongly in favour of the belief that such oligosaccharides serve as a temporary reserve of readily-available carbohydrate. Nevertheless, it is interesting and possibly significant to observe that the relationship between fructosans and water-soluble pentosans, first referred to by MacLeod & Preece (1954) in the cereals, has been found generally to hold throughout the species now investigated. Although small amounts of water-soluble pentosans were detected in all samples, appreciable quantities were generally present only in seeds in which fructosans were also detected. There has been much investigation and speculation concerning the nature and significance of fructosans (Archbold, 1940; Whistler & Smart, 1953) and although little doubt remains that they serve as temporary carbohydrate reserves, it has been suggested that in some respects they are the precursors of starch. At present, it would clearly be unwise to dismiss summarily a possible relationship between fructosans and the water-soluble pentosans.

Following this reference to fructosans, it seems

appropriate to consider more fully here the carbohydrate content of seeds during the final stage of ripening. In discussing sugar contents it has already been pointed out that the evidence available suggests that the seed carbohydrates are not greatly dependent on the carbohydrate content of the vegetative parts of the plant and moreover, that different systems, presumably enzymic in nature, are responsible for the formation of the different sugars present in seeds. Some doubt must remain concerning the fructosans, for the information available shows that fructosans decrease in both the ears and stems during ripening, and therefore it might be assumed that at ripeness fructosans if still detectable will be merely a remnant of what previously had been present. But, for the reasons discussed previously in some detail, it is thought that the fructosans, and in general the carbohydrates, of the seeds are not simply supplied from the vegetative parts of the plant. It has also been pointed out that although some of the carbohydrate differences in the seeds of various species may be accounted for by the occurrence of "ripeness" at slightly different stages in the life of a plant, this factor is not sufficient to explain the carbohydrate differences completely.

With regard to those different carbohydrate contents, it is interesting to recall that each member

of the Hordeae examined contained fructosans and that apart from these, fructosans were detected only in the Bromeae. Little more need be said regarding the value of carbohydrate content to classification of the grasses except perhaps to make the somewhat amusing observation that in the tribe Hordeae, which is particularly homogeneous, only Hordeum, on the strength of its β-glucosan content, is a defaulter, and furthermore that Festuca (along with Lolium) is anomalous in the Festuceae by containing the raffinose isomer.

Of the polysaccharides included within the scope of this work, two molecular species of the hemicellulosic type were obtained namely, β-glucosan and arabo-xylan, and although much information is still lacking concerning those materials, it has generally been assumed that primarily they serve a structural function. In addition, the method of aqueous extraction invariably yielded quantities up to 0.5% of a glucosan, starchy in nature, and it is perhaps this material whose significance is most imperfectly understood. As already stated, while resembling starch closely, it is extracted at a temperature lower than that which would normally extract starch and several possible reasons for this behaviour present themselves. It may be that this glucosan resembles glycogen more closely than most

plant starches, or perhaps simply that the effect of grinding the seeds is to affect a number of starch grains in such a way that the starch is more readily extracted, or finally it is possible that this material is not in fact contained within the starch grains.

Some indication of the probable function of the hemicellulosic polysaccharides is obtained by a microscopic inspection of the sections of various species. Plate VII shows the striking difference in endospermic structure which exists in different species. Within the endosperm of Bromus seeds, which are perhaps the most striking, the starch grains are easily distinguished surrounded by cell-walls. Those cell-walls are unusually thick and distinct, the walls of adjacent cells giving the impression of being cemented together. It would appear that within each cell, the starch grains are not simply packed together, but that they are embedded in a matrix which gives the impression of being not entirely distinct from the cell-walls. There seems little doubt that the hemicellulosic material extracted by water, and much of that extracted by dilute soda, is a constituent of those cell-walls; it may also be that some of it is a constituent of the matrix which appears to surround the starch grains. Presumably some quantity of cellulose is present in the cell-walls

but it is impossible, at present, to suggest how this cellulose and the hemicelluloses may be associated. In this connection it is of interest to note that preliminary observations of the effect of a β-glucosanase on transverse sections of Bromus seeds have revealed that such an enzyme preparation can rapidly separate the coherent endosperm into individual cells. This enzyme had no similar effect on turnip which has a high content of pectin. Although pectic materials form the middle lamella in tissues such as wood cambium and young roots it may well be that β-glucosan acts as an intercellular cement in Bromus endosperm in place of, or in addition to, the derivatives of polygalacturonic acid fulfilling such a function in meristematic tissues.

It is perhaps worth referring to the emphasis accorded to investigation of Bromus seeds during this work. Not only do they contain a somewhat unusual range of sugars, but more important, the extremely high quantity of gum and hemicellulosic carbohydrate present in those seeds affords the opportunity of studying those materials in some detail. Although all grass seeds probably provide a reasonable yield of hemicelluloses of the husk-type, it now appears that it is only a somewhat restricted number of species which contain appreciable quantities of endospermic hemicellulosic carbohydrate (of which the water-soluble

gums constitute a considerable proportion). Seeds of the Bromeae provide what appears to be the richest source of this material.

Of the relationship existing between the non-starchy polysaccharides of the seed, the virtual identity of water-soluble gums and much of the hemicellulose extracted by dilute soda has already been discussed. It appears however that a real difference does exist between the pentosans of the endosperm and of the husk. Those do in fact have a similar structure and the interchange from one type of molecule to another by metabolic processes such as transglycosidation of arabinose residues from one xylan chain to another is under consideration. It is, however, largely a matter of speculation at the moment as to the relationship if any between those molecular entities.

Most of the information which has previously been obtained concerning the chemistry and biochemistry of the carbohydrates of the Gramineae was from a restricted number of grasses, mainly the cereals. The earlier work of de Cugnac (1931) and then that of Archbold (1940) resulted in a reasonable understanding of the carbohydrate changes during growth of the vegetative parts of the plants, and more recently the problems arising from interest in silage have called for investigation of certain of the more common cultivated

grasses. As to the seed, the work of James (1940) and James & James (1940) on barley was the most comprehensive study before the advent of paper chromatography. More recently, Preece et al. (1952, 1953, 1954, 1956, 1957), MacLeod et al. (1951, 1953, 1954) and the Canadian workers (Meredith et al. 1953, 1955) have attacked the general problem of cereal biochemistry with a considerable emphasis on barley. It was the purpose of this present investigation to gather information regarding a much greater number of members of the Gramineae and as far as possible to establish the degree of similarity, or otherwise, existing amongst the members of the grass family. It has become abundantly clear that while there is a wide measure of similarity with respect to carbohydrate content, striking differences also exist in a number of respects. The results suggest that while the metabolic processes in the seed during ripening and germination are in many cases similar, nevertheless important differences do occur. To gain a fuller understanding of such processes, investigation of the enzymes concerned may now be the most valuable approach to the problem. Investigation not only of the degradative but also synthetic enzyme systems which exist, when considered along with the information already obtained should go some considerable way to provide this understanding.

SUMMARY

1. A survey of the water-soluble carbohydrates of the seeds of 25 species of grasses has been carried out. Two groups of carbohydrates were investigated, namely sugars and oligosaccharides extracted by 80% ethanol and the polysaccharides extracted by water at 40°C. Considerable differences are found to exist qualitatively and quantitatively in the carbohydrates obtained from different grasses.

2. All the seeds investigated contained sucrose, glucose and fructose. In addition to those sugars, other oligosaccharides were present in all but one species, but those oligosaccharides differed considerably from species to species. The oligosaccharides which have been detected are raffinose, the homologues of raffinose, the low-molecular fructosans and a trisaccharide which is similar to raffinose and which apparently has not previously been reported. The structure of this trisaccharide has not been established by conclusive chemical methods, but the structure indicated by the information available is O-α-D-galactopyranosyl-(1→3)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside.

3. The polysaccharides extracted by water at 40°C. from different species amounted to as little as 0.1% and as much as 2%. The principal constituents are

β-glucosan and arabo-xylan (the gums) and a starchy-type glucosan. Small quantities of mannose and galactose were detected on hydrolysis but no definite evidence is available as to how they occur in the polymeric form. The starchy glucosan did not occur in any sample to more than approximately 0.5% but in a large number of cases it is the principal constituent.

4. It is suggested that the quantities and nature of these carbohydrates may depend to some extent on the stage at which "ripeness" occurs in the plant, but that different enzyme systems must exist to account for some of the carbohydrate differences.

5. The possibility has been investigated of classifying the grasses on the basis of the water-soluble carbohydrate content of their seeds. Six groups of species have been distinguished by their sugar content and six groups by their polysaccharide content. It is suggested that these groupings are of taxonomic value, particularly in the case of grasses about which doubt exists, on botanical grounds, as to their best classification. For example, on the basis of carbohydrate content, the Hordeae are a very homogeneous tribe while Bromus and Brachypodium which have been grouped together botanically, are quite distinct.

6. The hemicelluloses extracted by dilute soda were obtained from four of the seed samples namely Bromus, Elymus, Avena and Phalaris. The composition of these hemicelluloses supports the view that in many respects the water-soluble gums and some at least of the hemicelluloses are structurally very similar. This applies particularly to β-glucosan.

Non-starchy carbohydrates were also obtained from Elymus and particularly from Bromus mollis by aqueous extraction in an autoclave prior to soda extraction. Again, the carbohydrates appeared to be the same as the gums.

7. (a) During the germination of Lolium seeds, the concentration of the "raffinose isomer" fell to a very low value. It seems likely that this sugar is utilised in a way similar to the utilisation of raffinose in germinating barley.

(b) The seeds of Bromus mollis which had a starch content of 15% yielded approximately 14% of hemicellulosic material on extraction with water and dilute sodium hydroxide. When these seeds were germinated, the yield increased to 16% after two days but finally dropped to 8%, of which only 3% was β-glucosan. This glucosan loss represents over 60% of the maximum β-glucosan. These findings strongly suggest that the β-glucosan can be metabolised by the germinating seed.

Appendix I

Separation of Sugars on Paper Chromatograms

Certain practical difficulties, which are largely overcome by acquiring experience of the technique are inevitably encountered in the use of paper chromatographic methods. It seems worthwhile to refer to certain of the practical problems involved in the use of the technique for both qualitative and quantitative analysis. Such analysis was concerned with the identification and estimation of, firstly, the free sugars and oligosaccharides of the seeds, and secondly, the monosaccharides obtained by hydrolysis of polysaccharides.

Free sugars and oligosaccharides

Comparatively little manipulative difficulty was encountered. No. 1 or 3 MM paper was used for separations and butanol/acetic acid/water was used as solvent.

Monosaccharides produced by polysaccharide hydrolysis

Analysis of these monosaccharides presented more difficulty. The first and perhaps main problem was to neutralise the acid and remove sufficient of the salt so as to allow satisfactory separation of the sugars. Neutralisation of H_2SO_4 was effected by use of NaOH, the utmost care being taken to obtain a pH of approximately 6. It is particularly important to prevent the solution becoming alkaline in view of the

danger of Lobry de Bruyn transformation. Accordingly, the solution was almost neutralised by addition of N NaOH, and very dilute NaOH was added to the solution so that it was just alkaline to methyl red and consequently had a pH of approximately 6. It was thought that the use of methyl red rather than methyl orange, as indicator, subsequently permitted a more satisfactory chromatographic separation although no reason is obvious for this opinion.

After neutralisation and removal of the salt by precipitation using ethanol, the solution was concentrated and applied to paper. For quantitative analysis, it was found desirable to evaporate the solution to dryness, take up in a small volume of 50% ethanol with prolonged stirring, filter and concentrate again before applying to the chromatogram. In this way much of the small amount of residual salt was removed and consequently a much larger amount of sugar could be put on the paper.

By this procedure and by the use of 3 MM rather than No. 1 paper, it was possible to separate, very satisfactorily, quantities of approximately 3 mg. sugar mixture (after hydrolysis of 20 mg. polysaccharide). This mixture invariably contained glucose, arabinose and xylose, and in determining the percentage of each of those present, a total of 3 mg. allowed a titre difference (in the Somogyi method

using the iodimetric titration) of approximately 0.2 ml. to represent 1% of the total sugar. A fairly high degree of accuracy was thereby achieved.

The use of 3 MM paper had a further important advantage for quantitative analysis. There seemed virtually no tendency for the sugar bands to run unevenly down the paper, with the result that there was very little doubt that the sugar band would be located exactly between the reference spots run on either side of the chromatogram.

Finally, for hydrolysates in which arabinose and xylose were always detected, butanol/ethanol/water was used as solvent. This solvent is superior to many others in that good separation can be effected with a small amount of salt still present on the paper, and is superior to butanol/acetic acid/water in separating arabinose and xylose much more satisfactorily.

Appendix II

Quantitative Estimation of Sugars (Somogyi 1945)

Slight variation in the conversion factors for any sugar was found, but for any one preparation of the reagent, a linear relationship existed between the quantity of sugar and the volume of $Na_2S_2O_3$. For glucose, xylose and arabinose linearity was found to hold up to at least 3 mg. sugar.

The following average conversion factors were obtained. A value is quoted for ribose, obtained up to 1 mg. ribose.

0.140 mg. glucose	≡	1 ml.	0.005N	$Na_2S_2O_3$
0.134 mg. xylose	≡	"	"	"
0.149 mg. arabinose	≡	"	"	"
0.190 mg. ribose	≡	"	"	"

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